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(57) Abstract

The invention relates to a method of detecting, identifying and quantitating microorganisms, and to oligonucleotide probes for use in this method. In particular, the method relates to the typing of specific isolates of microorganisms, and discrimination between strains and allelic subtypes. The method uses amplification of the 16S-23S rRNA spacer region using a highly conserved region from the 3' end of the 16S-23S rRNA spacer region, and/or a highly conserved region from the 5' end of said region. The method enables epidemiological tracing of specific microorganism subtypes. Preferred primers are disclosed and claimed.

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TYPING OF MICROORGANISMS

This invention relates to a method of detecting, identifying and quantitating microorganisms, and to oligonucleotide probes for use in this method. In particular, the method relates to the typing of specific isolates of microorganisms, and discrimination between strains and allelic subtypes.

Background and Prior Art

Correct identification of microorganisms is a vital part of microbiological practice, and is a prerequisite for selecting the most suitable form of treatment of disease, for prevention of contamination of foods, and for prevention of cross-infection. In many cases it is important not only to identify the species of organism, but also to determine the strain and serotype, or even an allelic subtype. Such identification at sub-species level is particularly important in epidemiological tracing, for example in establishing the origin of hospital-acquired (nosocomial) infection.

Disease caused by *Staphylococcus aureus* is most often the result of hospital-acquired infections. Strains of *S. aureus* that are resistant to the penicillinase-resistant antibiotic methicillin are now common, the first major nosocomial epidemic of a methicillin-resistant strain of *S. aureus* (MRSA) having been described by Stewart and Holt (1963). The determination of whether or not isolates of *S. aureus* represent a single strain is of considerable epidemiological value in a hospital setting.

Conventional methods of microbiological identification require culturing of microorganisms in a suitable growth medium, and this entails a delay of at least 24 hours, and often much longer. The methods utilised are completely manual, and rely very heavily on the experience and skill of the microbiologist. Such methods do not lend themselves to automation.

The only alternative presently widely used is immunological identification, which usually requires the use of monoclonal antibodies. A prerequisite for such immunological identification is that the species of the organism in question be known, or at least strongly suspected. Some antibodies of broad specificity are available for use in preliminary screening. While immunological methods can be automated, they are time consuming and expensive.

Other typing methods, which can be used for certain species only, include toxin detection, isolation of plasmids, bacteriophage, bacteriophage/bacteriocin typing systems, antibiotic susceptibility testing, protein typing by SDS-polyacrylamide gene electrophoresis, pulsed-field gel electrophoresis, immunoblotting, and restriction endonuclease analysis.

The availability of molecular biological methods, including oligonucleotide probing and polymerase chain reaction (PCR), offers a means of more accurate, and more rapid and precise identification. It also permits the identification of previously unknown organisms. Techniques based on analysis of DNA are more discriminating than traditional methods, and overcome the variability inherent in discriminating between strains by assays which rely upon the phenotype of the target organism.

The rRNA operon, *rrn*, is present in varying copy number in all bacteria, with some regions highly conserved and others highly variable (Neefs *et al*, 1990). Consequently, when genomic DNA digested with a restriction enzyme is hybridized to rRNA operons, several bands are detected (Garnier *et al*, 1991). The Southern hybridization of rRNA operons (ribotyping) to detect restriction fragment length polymorphisms (RFLPs) between strains has been reported in many bacterial species, including *Salmonella typhi* strains (Altwegg *et al*, 1989), *E. coli* strains (LiPuma *et al*, 1989), *Xanthomonas maltophilia* (Bingen *et al*, 1991), *Legionella pneumophila* strains (Harrison *et al*,

1992), and *Staphylococcus* species and subspecies, including MRSA strains (Blumberg *et al*, 1992; Monzon *et al*, 1991; Preheim *et al*, 1991; DeBuyser *et al*, 1992). However, Southern hybridization is slow and labour intensive.

Although the rRNA operon has a very high genetic stability and the length of the 16S rRNA gene is constant in all eubacteria (Neefs *et al*, 1990), the number of rRNA operons has been completely analyzed by Southern hybridization or PCR of the rDNA in only a few eubacteria, including *Escherichia coli*, demonstrating 7 operons (Morgan *et al*, 1977), *Bacillus subtilis*, 10 operons (Loughney *et al*, 1982), *Clostridium perfringens*, 10 operons (Garnier *et al*, 1991), *C. difficile*, 10 operons (Gürtler, 1993) and *Mycobacterium* species (Bercovier *et al*, 1986) and *Mycoplasma* species (Amikam *et al*, 1984) 1 or 2 operons respectively. The reports describing sequence data for the 16S-23S spacer region all include only a part of the total number of rRNA operons per genome, including *E. coli* demonstrating 5 spacers (Harvey *et al*, 1988), *B. subtilis*, 2 spacers (Loughney *et al*, 1982), *Pleisomonas shigelloides*, 3 spacers (East *et al*, 1992), *Aeromonas hydrophila*, 3 spacers (East & Collins, 1993), *Caulobacter crescentus* (Feingold *et al*, 1985), *Acholeplasma laidlawii* (Nakagawa *et al*, 1992) and *Enterococcus hirae*, (Sechi & Daneo-Moore, 1993) 2 spacers respectively.

International Patent Publication No. WO 91/16454 by N.B. Innogenetics S.A. describes use of hybridization probes consisting of at least 15 nucleotides from the spacer region between rRNA genes of non-viral organisms for detection of non-viral microorganisms, particularly bacteria. The probes are species-specific, and are preferably 15 to 100 nucleotides of the spacer region between the 16S and 23S rRNA genes. A separate oligonucleotide probe is required for each microorganism species.

U.S. Patent No. 5,288,611 by Kohne describes methods and probes for identification and quantification of

any organism or group of organisms containing rRNA, including previously unknown organisms. Probes specific for individual species and for groups of related species, and Probes hybridizing to rRNA or to tRNA are described.

U.S. Patent No. 5,292,874 by Milliman discloses hybridization probes specific for *Staphylococcus aureus* probes, which detect a unique rRNA sequence in the 23S rRNA gene.

Japanese Patent Publication No. 6090793 by Takara Shuzo Co. Ltd. describes methods for detection of bacteria of the genus *Lactobacillus*, by detection of a sequence in the spacer region between the gene encoding 16S rRNA and the gene encoding 23S rRNA.

The disclosure of each of these patent specifications is incorporated herein by reference. In each case sequences within the spacer region were selected on the basis of specificity for the organism from which they were isolated, and used in hybridization assays or polymerase chain reaction (PCR) for specific identification of an organism. Each of U.S. Patent No. 5,288,611, U.S. Patent No. 5,292,874 and Japanese Patent Publication No. 6090793 requires a separate oligonucleotide for each species of organism. None of these specifications mentions the existence or number of *rrn* alleles, or describes a method permitting differentiation of strains within a species, or of allelic variations.

International Patent Publication No. WO 93/11264 by E.I. Du Pont De Nemours & Company, the contents of which are incorporated herein by reference, discloses a method for identification of microorganisms by amplification of hypervariable spacer regions between highly conserved sequences encoding rRNA. These spacer region sequences are amplified, using primer sequences; the same pair of primers is used for all species of microorganisms; the sequences of these primers are highly conserved among prokaryotic organisms. The products of amplification are characteristic of a given species. A further amplification

step using arbitrarily primed polymerase chain reaction (AP-PCR; also known as randomly amplified polymorphic DNA; RAPD) is described as being able to differentiate serotype or strains within a species. However, it is evident that this method did not always enable differentiation between strains, and even if this differentiation was achieved, the patterns were not always clear. The conserved regions used as primers in WO 93/11264 are designated E and A herein, as described below. These findings have also been published elsewhere (Jensen *et al*, 1993; Jensen & Straus, 1993). The contents of these publications are also incorporated herein by reference.

PCR analysis of the 16S rRNA gene has been used to demonstrate species-specific differences (Gürtler *et al*, 1991) and strain differences (Vaneechoutte *et al*, 1992) in various bacterial species. Allelic species-specific differences within the 16S rRNA gene have been demonstrated in clostridia (Gürtler *et al*, 1991). The rRNA alleles of *E. coli* (Brosius *et al*, 1981) and *B. subtilis* (Loughney *et al*, 1982) have been shown to have variable length 16S-23S rRNA spacer regions.

We have now surprisingly found that the presence or absence of specific variable length rDNA spacer regions varies between strains within a given microorganism species. By using different specific conserved regions of the rRNA operon, designated C and D herein (see Figure 1 below), as primers for polymerase chain reaction, and by using modified PCR conditions, we can achieve amplification of all alleles present in a microorganism sample, and thus we can differentiate between strains in any bacterial species, without the need for any further steps. The patterns obtained were stable within strains on repeated testing, using passaging either *in vitro* or *in vivo*, permitting discrimination within and between species, and designation of specific types within strains.

Brief Description of the Drawings

Figure 1 shows the position of the oligonucleotide primers (A, B, C, D, E) used in the prior art and in the present invention. The abbreviations ile and ala refer to the respective genes-encoding tRNA for isoleucine and alanine. Primers A and E are as disclosed in WO 93/11264.

Figure 2 illustrates the approaches used for the detection of rRNA alleles in *C. difficile* by Southern hybridization and PCR. The hatched bars (A, B, C and D) show positions of the respective PCR products (Table 3), the shaded bar denotes the 16S rRNA gene, the solid bar denotes the 23S rRNA gene, and the line joining the 16S and 23S gene depicts the spacer regions. The *Hin*dIII site is at position 975 of the 16S rRNA gene (Gürtler *et al*, 1991).

Figure 3 shows hybridization of PCR product B to Group II bands in genomic DNA isolated from *C. difficile* and *C. bifermentans* strains.

Lanes 1-10, *C. difficile* strains H13, H15, H16, H17, H18, H19, H20, H23, 9689 and 9689, respectively;

Lanes 11-13, *C. bifermentans* strains AM312, AM360, and AM818, respectively;

Lane 14, pBR328 DNA digested with *Bg*II and *Hin*fI, labelled with photodigoxigenin;

Y indicates the position of an extra band visible in *C. bifermentans* products.

Figure 4 illustrates the hybridization of PCR product A to Group I and II bands in genomic DNA isolated from *C. difficile* strains.

Lane 1, pBR328 DNA digested with *Bgl*I and *Hin*fI, labelled with photodigoxigenin;

Lanes 2-11, H24, H25, H26, H27, H28, H29, H30, H31, H32 and H33, respectively.

Figure 5 shows the detection of rRNA alleles in *C. difficile* strains by Southern hybridization. The symbols, box shadings and the position of the *Hin*dIII site are described in the legend to Figure 2. Bands depicted as

Group I (Figures 3 and 4) correspond to fragments 5' of the *Hin*dIII site and Group II bands (Figures 4 and 5) correspond to fragments 3' of the *Hin*dIII site. ★ refers to bands which are not present in all strains.

Figure 6 shows the constant and variable length regions within PCR product C amplified from *C. difficile* strains, as demonstrated by agarose gel electrophoresis of undigested (lanes 2-7) and *Hin*dIII-digested (lanes 10-15) PCR product C.

Lane 1, pBR328 DNA digest with *Hin*fI and *Bgl*I;

Lanes 2-7, H15, H24, H28, H30, H31 and H33, respectively;

Lane 8, no DNA control;

Lane 9, pBR328 DNA digested with *Hin*fI and *Bgl*I;

Lanes 10-15, h13, H14, H17, H19, H23 and 630, respectively.

The standards are 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234 and 220 bp respectively.

C_d, constant *Hin*dIII-digested;

V_u, variable undigested; and

V_d, variable *Hin*dIII-digested.

Figure 7 shows denaturing PAGE of PCR product C amplified from strains of *C. difficile*.

Lanes 1-8, H17,H15, H14, H13,, 6989, 630 H23 and H19, respectively;

Lane 9, no DNA PCR control

Lanes 10-19, H33, H32, H31, H30, H29, H28, H27, H26, H25 and H24, respectively.

The sizes of the respective alleles are shown on the left [mean ± SEM (number of determinations)]. The molecular mass markers used (not shown) were λ DNA digested with *Hin*dIII and *Eco*RI (947 and 831 bp bands only) and SPPI DNA digested with *Eco*RI (1150 and 1000 bp bands only).

Figure 8 is a dendrogram showing the relationships of *C. difficile* ribotypes. Using maximum parsimony, 50 equally parsimonious trees were found, one of which is shown. The same ribotypes were found in each of

the circled branches (*a, b, c*) for all 50 trees. The root of the tree (*C. bifermentans*) had no bands in common with any of the ribotypes.

Figure 9 shows the distribution of rRNA genes and restriction sites in the region of interest in *Staphylococcus aureus*. The solid line joining these genes can vary in length in the same strain or in different strains (Figure 12b). At the bottom of the diagram, the dashed lines show positions of the PCR products C, I & J, which were obtained using the primers R1392F and LR488, SP1F and SP2R and SP1F and LR20F respectively (Table 8). The dotted lines show the origins of *Hpa*II fragments (E, F, G and H) obtained from PCR product C. The locations of other primer binding regions that were used to sequence *Hpa*II fragment E are also shown.

Figure 10 illustrates denaturing PAGE of PCR products amplified from strains of *S. aureus*.

(a) Methicillin resistant *S. aureus* (MRSA) ribotype A, (lanes 1-3), and ribotype B (lanes 4-8). The sizes of the respective alleles are shown on the right [mean ± SEM (number of determinations)].

(b) All MRSA ribotypes A-I (excluding C) and penicillin-sensitive ribotype Pa (ATCC 9144).

(c) Penicillin and methicillin sensitive *S. aureus* strains.

Lanes 1-6: Ribotypes Pi, Pj, PF A(strain H11);
Lanes 9-16: B, Mi, Mh, Mi, Pi, Mh and Mj.

The sizes of the respective alleles are shown on the right [mean ± SEM (number of determinations)]. The molecular mass markers (lanes 7 & 8) used were λ DNA digested with *Hin*dIII and *Eco*RI (1375 and 947 bp bands only) and SPP1 DNA digested with *Eco*R1 (1150 and 1000 bp respectively).

Figure 11 shows the alignments of 16S-23S spacer sequences from *S. aureus*. PCR product C from *S. aureus* strains (Table 9) was cloned into M13mp18RF and sequenced with the primers listed in Table 8 and Figure 9. The

sequences were derived from the clones and isolates listed in Table 7. The sequences SA16S and SA223S were taken from Ludwig *et al* (1992). The alignment of *rrn* alleles with SA16S (a), *rrnC*, E, F, G, H, J, K & L (b) and *rrn* alleles SA23S (c) is shown. The symbols refer to an identical base (.), and absent base (-), † = (*rrnA*, E, J, L, F), * = (clones 4, V17, V32) and § = (clones 4, V4, V8, V32, V43).

Figure 12 is a dendrogram showing the relationships between the 16S-23S alleles using the data in Figure 13b or Table 7. The same tree was obtained using either sets of data. One parsimonious tree was obtained with the program DNA PENNY from the PHYLIP package. Then, using MACCLADE, of the 16 possible rerootings, the tree shown was selected because it was drawn using the longest allele (*rrnA*) as the root. The numerals indicate the numbers of changes/branch. The dotted lines show the clades which contain alleles without tRNA genes, and the solid lines show the clades which contain alleles with tRNA genes.

<u>Summary of the Invention</u>

The method of the invention avoids cumbersome steps required by previously available methods, and is suitable for testing large numbers of samples; it is also amenable to automation.

The method of the invention is particularly suitable for epidemiological studies, for example identifying the source of hospital outbreaks of antibiotic-resistant microorganisms, or tracing the source of microorganisms causing contamination of foodstuffs.

According to a first aspect, the invention provides a method of identification of microorganisms, comprising the steps of extracting and purifying DNA from a sample suspected to contain bacteria, and subjecting the 16S-23S rRNA spacer region of said DNA to amplification, using a first primer comprising a sequence from the 5' end

of the 16S rRNA gene, and a second primer comprising a sequence from the 3' end of the 23S rRNA gene, thereby producing fragments having detectable differences in size and number, and separating the amplified fragments.

Because the amplified fragments produced in the method of the invention are of variable length, they can be analysed directly, for example by electrophoresis; no other experimental step, such as hybridzation, is necesary in order to demonstrate differences between strains, although in some situations a hybridization step could be advantageous.

The amplified products may be separated by any method which provides sufficient resolution. We have found that small conventional polyacrylamide gels have somewhat poor resolution, and our studies have used long denaturing polyacrylamide gels. However, the person skilled in the art will be aware that other separation methods, such as capillary electrophoresis or high performance liquid chromatography, may be used.

Similarly, the studies described herein have employed amplification of DNA sequences by polymerase chain reaction. However, the skilled person will be aware that other amplification methods are known, and may also be used. For example, ligase chain reaction, 3SR amplification, strand displacement amplification, Qβ replicase reaction, or branched DNA signal amplification are available (see Wolcott, 1992 for review). The skilled person will readily be able to test these alternative methods for suitability for use in the invention.

Optionally additional probes may be used, for example comprising the sequence encoding $tRNA^{ile}$ and/or the sequence encoding $tRNA^{ala}$.

The sample will usually be a clinical sample such as blood, tissue, urine, faeces, sputum *etc.*, a food sample, or an environmental sample such as a water sample or a soil sample. Other types of samples may be used, depending on the circumstances.

The DNA may be extracted by any suitable method, but preferably the method is a rapid one. Extraction with guanidine hydrochloride or by boiling water followed by column purification are both suitable. In some cases, particularly where clinical specimens are used, it may be advantageous to effect a preliminary purification of the sample following DNA extraction. If the nature of the bacteria sought to be tested is known, this may be carried out using monoclonal antibody methods, such as those using monoclonal antibody conjugated to magnetic beads. Some broad spectrum antibodies are also available for this purpose.

While the method of the invention is specifically described with reference to *Clostridium difficile* and to *Staphylococcus aureus*, it will be clearly understood that the invention is not limited to these organisms, and is applicable to any microorganism for which the sequences of the 16S rRNA gene and the 23S rRNA gene are known. These sequences enable suitable probes to be designed.

Preferably the primers used correspond to a highly conserved region from the 3' end of the spacer region, and to a highly conserved region from the 5' end of the spacer region respectively.

According to a second aspect, the invention provides amplification primers for use in the method of the invention. As described above, these primers correspond to highly conserved regions from the 3' end of the 16S-23S rRNA sacer region and the 5' end of the 16S-23S rRNA spacer region respectively. Preferably they correspond to regions from the 5' end of the 16S rRNA gene and to a region from the 3' end of the 23S rRNA gene respectively. Preferably the primers are 15 to 20 nucleotides long, since 15 nucleotides is generally considered to be a minimum length of primer for PCR, but conservation is generally lost at greater than 20 nucleotides.

In a preferred embodiment the primers are R1391F and LR488 or LR194F as herein defined. Most preferably LR488 is 15 to 19 nucleotides long, and R1391F is 15 to 18 nucleotides long. Primer C (LR488) is particularly preferred, because it is more highly conserved than primer A.

We have found that in fact there are three regions in the first 520 base pairs of the 23S rRNA gene which are highly conserved through a wide variety of species of bacteria and fungi, and which are useful as amplification probes in the present invention. These are summarised in Table 1.

Table 1

Conserved regions in the first 520bp of the 23S rRNA gene

Species	Position 21 to 38§ (A)	Position 188 to 208§ (B)	Position 456 to 474§ (C)
E. coli Plus strand	A-CGGTGGATGCCCTGGCA	GAACTGAAACATCTAAGTACC	CAGTACCGTGAGGGAAAGG
Compementary strand	T-GCCACCTACGGGACCGT	CTTGACTTTGTAGATTCATGG	GTCATGGCACTCCCTTTCC
Pseudomonas aeruginosa	.-...........T.....	....	....
Pseudomonas cepacia	G-T..........T....G	...................A.	....
B.acillus subtilis	.-...........T.....	....	....
Bacillus stearothermophilus	.-...........T.....	..............T......	....
Staphylococcus aureus	.-...........T.....	..............T......	....
Staphylococcus carnosus	.-...........T.....	..............T......	....
Streptococcus haermophilus	.-...........T.....	...................G.	....
Neisseria gonorrhoeae	-CA..C.......T....G	........C............	....
Listeria monocytogenes	.-...........T.....	....	....
Frankia sp.	.-...........T.....	...G..........C.T....	T..................
Mycobacterium leprae	.-T..........T.....	...G..........C......	................T..
Rhodopseudomonas capsulata	T-T..........T.....	....	....
Rhodobacter sphaeroides	--T..........TA....	....	....
Rum.amylophylus	.-T..........TA....	...G..........C......	A..................
Streptomyces griseus subsp.*griseus*	.-...........T.....	...G..........C......	T...............T..

§ Numbering of nucleotides based on the *E. coli* 23S rRNA gene [Brosius, 1980 #126]
(.) refers to nucleotide identical to plus strand of *E. coli*.

Detailed Description of the Invention

The invention will now be described by way of reference only to the following non-limiting examples, and to the drawings referred to above

Bacterial strains and their Cultivation

Bacterial strains used herein are listed in Tables 2 and 3.

The identity of all strains of *Clostridium difficile* was determined by biochemical tests (Cato *et al*, 1986) and confirmed by gas-liquid chromatography (Sutter *et al*, 1985). Purified stocks were stored in cooked-meat broth at room temperature or in glycerol broth at -20°C. All strains were grown in brain heart infusion broth (BHI, Gibco). The stability of ribotype patterns was tested by passaging single colonies from horse blood agar plates every 2-3 days over a 5 week period. Toxin B production by *C. difficile* strains was detected by the method of Boondeekhun *et al* (1993).

The identity of all *S. aureus* strains was determined by biochemical tests (Kloos & Schleifer, 1986), and antibiotic sensitivity tests were assessed by the agar dilution method (break points were determined according to NCCLS guidelines, Vol 13 No. 25, 1993). Purified stocks were stored in glycerol broth at -70°C. All strains were grown in trypticase soya broth (TSB,Oxoid). The stability of ribotype patterns was tested by passaging single colonies from sheep blood agar plates five times a week for six weeks.

Table 2

Strain	T	RT	Plasmid	Year of isolation	Source
C. difficile					
H17,H18	+	G	-	1990	
H23	+	F	-	1990	
H24	+	J	ND	1990	
H25	+	D	ND	1990	
H26	-	K	ND	1991	
H27	+	G	ND	1990	Heidelberg Repatriation Hospital, Melbourne, Australia
H28	+	L	ND	1990	
H29	+	H	ND	1991	
H30	+	M	ND	1991	
H31	+	D	ND	1991	
H32	+	N	ND	1991	
H33	+	E	ND	1991	
H[illegible]4,H16,H20	+	E	+ (A)	1990	
H13	-	O	-	1990	
H14	-	P	+ (B)	1990	
H15	+	D	-	1990	
6390	+	H	ND	1993	
6048	+	G	ND	1993	
AM690	ND	Q	ND	1982	St Vincent's Hospital, Melbourne, Australia
ATCC 9689	+	R	-		American Type Culture Collection
630	+	F	+ (C)	1987	H. Hächler, Switzerland
C. bifermentans					
ATCC 638					American Type Culture Collection
AM312					Dr R. Wilkinson, University of
AM360					Melbourne, Australia

Abbreviations: T Toxin production

RT PCR-ribotype of *C. difficile strains* (Table 5)

The plasmid types are designated:

- none detected

+ extrachromosomal bands detected (A, B and C are the different sized band paters obtained)

ND not determined.

Table 3

S. aureus strains

Location	Year	Number of Isolates
Methicillin-resistant		
Guildford, NCTC 10442	1960	1
New York, ATCC 33952	1981	1
Melbourne, RMH†	1982	10
UK, NCTC 11940	1982	1
UK, NCTC11939	1982	1
Melbourne, HRH	1982	6*
Ireland	1982-83	9
London, RFH	1983	8
London, NCTC 12232	1986	1
Melbourne, HRH	1988-89	7
Melbourne, HRH	1992-93	226§
Melbourne, RCH	1993	3
Penicillin-sensitive		
HRH	1992-94	14
Oxford, UK, ATCC 9144	1944	1
Bundaberg, Australia, NCTC 2669	1928	1
UK, NCTC 8532	1953	1
Methicillin-sensitive		
HRH	1992-93	31

The numbers of strains isolated from various locations at various times are shown. The strains from Ireland are listed in Townsend *et al* (1987) as WG1761-3 (plus 6 other strains) while those from RFH are listed in Townsend *et al* (1984) as WG2710, 2715, 2720 and 2724 and in Townsend *et al* (1987) as WG2716 (plus 3 other strains)

NCTC National Collection of Type Cultures, UK
ATCC American Type Culture Collection

HRH	Heidelberg Repatriation Hospital
RMH	Royal Melbourne Hospital
RCH	Royal Children's Hospital, Melbourne
RFC	Royal Free Hospital, London
*	including H11, H12, H14
$	including D46
†	including H21

DNA isolation and amplification

Genomic DNA and plasmid DNA was isolated by the protocol of Gürtler *et al* (1991), except that the cell walls of *S. aureus* were disrupted by incubating the strain with 200 g lysostaphin ml^{-1} at 37°C for 5-10 min. DNA regions (Figures 1 and 9) were amplified by the protocol of Gürtler (1991), except that the reaction volume and amount of DNA were halved, and 1.25 units *Taq* polymerase (Boehringer) were used. The primers used are shown in Tables 4 and 7 below. For the amplification of M13 clone inserts in *S. aureus*, 50-100ng of single stranded M13 clone DNA was added to PCR mixtures using primers M13F and R.

Restriction enzyme analysis

For *C. difficile*, purified PCR products R907-LR507 and R1391-LR507 were digested singly or doubly with 10-15 units *Hind*III and *Cfo*I, as instructed by the manufacturer (Boehringer). Genomic DNA was digested with 30 units *Hind*III. The digested and undigested PCR products were resolved on 2% (w/v) low-gelling-temperature plus 2% (w/v) 'AR' agarose gels. The *Hind*III-digested genomic DNA was resolved on 1% (w/v) 'AR' agaorse gels.

For *S. aureus*, PCR product M13F-M13R was digested with 10-15 units *Dra*I or *Hinf*I, as instructed by the manufacturer (Boehringer). Genomic DNA was digested with 20 units *Hpa*II. The digested PCR products and genomic DNA were resolved on 2% w/v low-gelling-temperature plus 2% (w/v) 'AR' agarose gels.

Southern hybridization

The protocol of Gürtler *et al* (1991) was followed. PCR products were labelled with digoxigenin.

Denaturing PAGE

The amplification protocol described above was followed with some modifications. The reaction volume was decreased by a factor of two and 2 µCi [α-^{32}P]dATP (DuPont or Amersham) was added. The reduction of the unlabelled dNTPs by a factor of four increased the yield of labelled product. Radiolabelled DNA fragments were separated on a 0.4 mm thick, 38 cm wide and 50 cm high (Bio-Rad), 3.5% (w/v) polyacrylamide gel containing 7 M-urea (Sambrook *et al*, 1989). Gels were dried in a vacuum slab gel drier (Bio-Rad) for 2 h at 80°C. Autoradiographic exposure was 18-96 h.

DNA sequencing

Sequencing was performed by the dideoxynucleotide method of Sanger *et al* (1977) using the *Bst* DNA sequencing kit (BioRad). 7-Deaza-2'-deoxyguanosine triphosphate was used to minimize band compression due to GC-rich regions. The primers used for sequencing are listed in Table 8 below and in Figure 9.

Data analysis

DNA sequences were processed and analysed by the following methods. The DNASIS program (version 6; Pharmacia) was used to orient, join and edit DNA sequences. The orientation of inserts was deduced by alignment with the 16S or 23S rDNA seqences from *B. subtilis* (Green *et al*, 1985) or *S. aureus* (Ludwig *et al*, 1992). The 17 sequences (fragment E, Figure 1) were aligned using CLUSTAL V (Higgins *et al*, 1992) and were aligned to the 16S and 23S rDNA sequences from *S. aureus* (Ludwig *et al*, 1992). Further modifications to the alignment were done using MACCLADE software (Maddison & Maddison, 1992).

Phylogenetic analysis was done with the software package PHYLIP using DNAPENNY (Felsenstein, 1993). The resulting treefile was then imported into MACCLADE for further analysis and presentation.

The presence or absence of PCR product C bands was analysed as follows:

(1) The presence or absence of bands (corresponding to region R1391-LR507) on autoradiograms was analysed by using the program BioImage (Millipore). The average sizes of the 16 alleles (*rrn*A-P) were calculated from five separate gels ranging from 1-51 determinations for the respective alleles. Using these sizes as internal standards, molecular masses were assigned to respective bands from the different strains. Twenty-four strains from four gels were then compared at once. Presence or absence of bands was scored by a 1 or 0, respectively.

(2) The resulting data matrix prepared from four gels was analysed by maximum parsimony (Swofford, 1985).

Intense bands were reported as positive; when the faint bands were also reported as positive the results did not change. The resulting data matrix was analysed using MIX and DOLLOP in the program PHYLIP.

<u>Example 1</u> <u>Ribotyping of Strains of *Clostridium difficile*</u>

The DNA typing approaches used are shown in Figure 2 and Table 4. Products A and B were hybridized to *Hin*dIII-digested genomic DNA isolated from *C. difficile* and *C. bifermentans* strains. Differences in *Hin*dIII sites on both flanking sides of the 16S rRNA gene were sought within and between strains. Products C and D were amplified from *C. difficile* strains in an attempt to find differences in the length of the 16S-23S spacer region within and between strains.

Table 4

Regions of the rRNA operon amplified and their corresponding primers

PCR product	Gene	Region amplified	16S-23S spacer	Primer code	Primer position and direction	Sequence
A	16S	15(S)-1408(S)	-	R015 R1391*	15-27(F) 1408-1391(R)	GATCCTGGCTCAG GACGGGCGGTGTGTACAA
B	16S	907(S)-1408(S)	-	R907 R1391*	907-926(F) 1408-1391(R)	AAACTCAAATGAATTGACGG GACGGGCGGTGTGTACAA
C	16S and 23S	1392(S)- 507(L)	+	R1391F*(D) LR488 (C)	1391-1408(F) 488-507(R)	TTGTACACACCGCCCGTC CCTTTCCCTCACGGTACTG
D	16S and 23S	907(S)- 507(L)	+	R907 LR488 (C)	907-926(F) 488-607(R)	AAACTCAAATGAATTGACGG CCTTTCCCTCACGGTACTG

The products amplified cover the regions shown where S=16S rRNA gene and L=23S rRNA gene. The sense of the primers used is shown by R=reverse, F=forward and *=identical region, with R1391 being the complemnt of R1391F. The positions of all the primers are in regions which are highly conserved in eubacteria (Neefs *et al*, 1990; Guttell & Fox, 1988). The nucleotide numbering system is that of *E. coli* operon (Brosius *et al*, 1978). The positions of each product are schematically represented in Figure 2.

The bands detected by Southern hybridization (ribotyping) have been divided into Groups I and II, showing numerous Group II differences between strains and fewer Group I differences. Ribotyping of 21 isolates of *C. difficile* from the Heidelberg Repatriation Hospital and one from St Vincent's Hospital, Melbourne, Australia, produced 14 restriction fragment length polymorphism (RFLP) types, 10 of which are shown in Figure 4. There were 10 group I bands, demonstrating 10 rRNA alleles in *C. difficile*.

Products A and B consist only of parts of the 16S rRNA gene (Table 4). We have shown previously that the 16S rRNA gene is of constant length between alleles and strains of *C. difficile* (Gürtler *et al*, 1991). When PCR product B was hybridized to *C. difficile* genomic DNA, Group II bands hybridized predominantly (Figure 3), the Group I bands hybridized faintly, because product B included 62 bp 5' of the *Hind*III site (1/10 of product B). When product B was hybridized to *C. bifermentans* genomic DNA digested with *Hind*III (Figure 3), no Group I bands hybridized and an extra band appeared (Y) due to an extra *Hind*III site at position 675 of the 16S rRNA gene (Gürtler *et al*, 1991). When PCR product A was used as a probe (Figure 4), the Group I and II bands hybridized with equal intensity. The orientation of the Group I and II bands is as shown in Figure 5 because product B hybridized predominantly to Group II bands and because the *Hind*III site lies 62 bp

downstream from the 5' end of product B (Gürtler *et al*, 1991).

Example 2 **Identification of a Variable Length Region Between the 16S and 23S rRNA Gene of *Clostridium difficile***

From Figures 3, 4 and 5 it can be seen that the Group II bands consist of the spacer region and part of the 23S rRNA gene. These Group II bands were of variable length, which could be explained by the presence of either a variable *Hin*dIII site or of an insertion within the spacer or the beginning of the 23S rRNA gene. To determine which possibility was correct, we amplified PCR products C and D, both of which include the spacer regions (Table 4). When the product C primer combinations of Table 4 were used, several bands (V_u) of varying molecular masses were obtained from each *C. difficile* strain, as shown in Figure 6. The presence of bands varied from strain to strain. When V_u bands were digested with *Hin*dIII, a band appeared at 430 bp (C_d); this was of higher intensity than the digested variable length bands, V_d. Band C_d appeared in all the strains listed in Table 2 (results not shown). When the product D primer combination was used, the same *Hin*dIII band (C_d) was present, demonstrating that this band contains the 23S rRNA gene from position 80-507 (Table 5). The demonstration of band C_d shows that this region is of constant length between alleles. Taken with the Southern hybridization data of Figures 3 and 4, these results show that the variable length regions lie between the 16S and 23S rRNA genes. The exact base pair location of the spacer regions can only be determined when the separate alleles have been sequenced.

Table 5

Variable 16S-23S rRNA spacer regions in *C. difficile* strains

Allele	Restriction enzyme site (position, bp)	*C. difficile* ribotype														Band Frequency *
		Q 1	L 1	D 3	E 4	J 1	N 1	G 4	F 2	O 1	P 1	K 1	H 2	M 1	R 1	
		Constant region (16S rRNA)														
All	*Hin*dIII (1010)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
All	*Cfo*I (1100†)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		Variable region (16S-23S spacer region)														
*rrn*A		-	-	-	-	-	-	-	-	-	-	-	+	-	-	8.3
*rrn*B		-	-	+	+	+	+	+	+	+	-	+	+	+	+	87.5
*rrn*C		-	-	-	-	-	-	+	-	-	-	-	+	-	-	16.7
*rrn*D		+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
*rrn*E		-	-	-	-	-	-	-	-	-	+	-	-	-	-	4.2
*rrn*F		+	+	-	+	+	-	-	-	-	-	-	-	+	-	33.3
*rrn*G		-	-	-	-	-	-	-	+	+	-	+	-	-	-	16.7
*rrn*H		-	-	-	-	-	-	-	-	-	-	+	-	-	-	4.2
*rrn*I		+	-	+	-	-	-	-	-	-	-	-	-	-	-	16.7
*rrn*J		+	+	+	-	-	-	-	-	+	-	+	-	-	-	29.2
*rrn*K		-	-	-	-	-	-	-	+	-	-	-	-	-	-	8.3
*rrn*L		+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
*rrn*M		+	-	-	-	-	-	+	+	+	+	+	+	-	-	50

Allele	Restriction enzyme site (position, bp)	*C. difficile* ribotype														Band Frequency *
		Q 1	L 1	D 3	E 4	J 1	N 1	G 4	F 2	O 1	P 1	K 1	H 2	M 1	R 1	
*rrn*N		-	+	+	+	+	+	-	-	-	+	+	-	-	-	50
*rrn*O		+	+	+	-	-	-	+	+	+	+	-	+	+	+	70.8
*rrn*P		-	+	+	-	+	+	+	-	+	+	+	+	+	+	70.8
		Constant region (23S rRNA)														
All	*Hin*dIII (80)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

* Calculated by dividing the number of isolates with allele *rrn* by the total number of isolates

† Enzyme site reported preivously (Gürtler *et al*, 1991).

Example 3 **Improving Resolution of Bands**

The resolution of the variable length bands was low, as can be seen in Figure 6, and so it was decided to increase the resolution with long denaturing polyacrylamide gels. When this was done, the same amplification products, designated V_u in Figure 6 separated into between 5 and 9 bands per strain, with the presence of bands variable between strains. These results are shown in Figure 7. Each band was assigned as an allele, resulting in a total of 16 alleles (A-P) of variable length. The constant length regions within the 16S and 23S genes were partially characterized, and the results are summarized in Table 5. The variability in length was due to variable length 16S-23S spacer regions between alleles.

PCR product C was amplified from various *C. difficile* strains and separated by denaturing PAGE (Figure 7). The presence of variable length alleles (*rrnA-P*) is shown. The size of each allele is shown in Figure 7. The outer limits of the constant regions are depicted by restriction enzyme-cut sites (see Figures 5 and 6). The strain numbers corresponding to the ribotype are listed in Table 2. The number of isolates in each ribotype is listed below each letter. Th constant length regions were collated from results obtained in Figures 3, 4 and 6 and Gürtler *et al* (1991). The variable length regions wree collated using BioImage software from Figure 7 and three other denaturing polyacrylamide gels.

Example 4 **Relationship Between Ribotypes of *Clostridium difficile***

When all of the *C. difficile* strains listed in Table 2 were analysed, 24 strains were divided into 14 ribotypes, which are also shown in Table 5. The dendrogram depicted in Figure 8 shows that 3 clusters (a, b, c) are found in all trees analysed. Within ribotype G, 2 isolates were cultured from one patient at different times; within ribotype E, 3 isolates were cultured from one patient at

different times. All other isolates which had identical patterns (ribotypes D, E, F, G and H) were from different patients.

Example 5 Stability of Band Patterns in *Clostridium difficile*

The stability of band V_o sizes and patterns was investigated in detail by passaging five strains over a 5 week period. The alleles were scored as positive or negative by appearing visually identical and by having similar calculated molecular masses. The results, illustrated in Figure 7 and Table 5, show that both the band sizes and patterns were highly reproducible in five *C. difficile* strains. The band sizes and patterns of strains H23 and 630 were reproducibly identical.

Product C was amplified from various *C. difficile* strains and separated by denaturing PAGE. Accumulated values (mean±SEM) taken from five separate electrophoresis runs are shown. The data include runs (PCR, DNA preparations and electrophoresis) done over a 9 month period, as well as a stability testing experiment with the number of single colony passages per strain shown.

Table 6

Stability of product C bands from *C. difficile* strains

Alleles	Strain				
	630	9689	H14 (type E)	H15 (type D)	H23 (type F)
rrnA	1166±3	1163±4	1164±4	1163±5	1161±4
rrnB	1108±2	1100±4	1106±3	1108±4	1106±3
rrnD					
rrnE			1068±2		
rrnF					1052±2
rrnG	1050±2				
rrnH					
rrnI				1008±2	
rrnJ				992±2	
rrnK	978±2				975±1
rrnL	945±3	937±2	948±3	943±3	938±2
rrnM	928±3				922±2
rrnN			908±3	906±3	
rrnO	890±3	884±1		887±3	885±1
rrnP		851±1		853±3	
Statistics					
n	14	8	11	9	9
DNA preparations	5	5	4	4	5
Passages	14	14	10	10	14
PCR runs	10	5	4	4	6

The main finding of the present study was that the presence or absence of specific variable length rDNA spacer regions varied between *C. difficile* strains. The patterns obtained were stable within strains upon repeated testing after passaging *in vitro* and *in vivo*, allowed the designation of strains to specific types, enabled discrimination within and between species, and allowed for the easy testing of large numbers of strains. Thus the novel molecular typing method of the invention may be applied to epidemiological studies of *C. difficile*.

Since 500 bp of the 5' end of the 23S gene was amplified, it was possible that the observed heterogeneity of PCR products was due to an insertion within the first 500 bp of the 23S gene. This possibility is supported by several findings. At least one extra cleavage site has been reported in the large rRNA subunit of *Leptospira interrogans* (Hsu *et al*, 1990) and *Salmonella* species (Hsu *et al*, 1992), producing several fragments smaller than 23S and a 90 bp intervening sequence has been shown to be excised during large subunit rRNA maturation (Burgin *et al*, 1990). The results presented in this specification show that in *C. difficile*, 430 bp 3' from position 507 of the 23S rRNA gene was of constant length and the 16S-23S spacer DNA was of variable length between alleles.

The 16S-23S spacer regions of *B. subtilis* (Vold, 1985) and *E. coli* (Fournier & Ozeki, 1985) contain tRNA genes which vary in length from 75-90 bp. Of the 7 *rrn* operons in *E. coli*, all contain from 1-3 tRNA genes (Brosius *et al*, 1981), while in *B. subtilis* operons, two out of the three analysed sets of the 10 *rrn* have been shown to contain tRNA genes (Loughney *et al*, 1982). It is possible that the 16S-23S spacer regions in *C. difficile* characterized herein may contain tRNA genes.

<u>Example 6</u> <u>Ribotyping of Strains of *Staphylococcus aureus*</u>

Genomic DNA was isolated from *S. aureus* as described above, and amplified using the primers described in Table 6 and Figure 9.

Table 7

Sequencing primers

Code	Region	Position	Direction (sense)	Sequence
R1391FH‡	16S	1440-1417¶	F(+)	GGCCGGTTGTACACACCGCCCGTC
R1391F* (D)	16S	1440-1417¶	F(+)	TTGTACACACCGCCCGTC
SP1F	16S-23S spacer	51-70†	F(+)	ATTGTATTCAGTTTTGAATG
SP1R	16S-23S spacer	51-70†	R(-)	TTACTTACTTATCTAGTTTT
TRNAF	$tRNA^{Ile}$	100-120†	F(+)	ATAGCTCAGCTGGTTAGAGC
TRNAR	$tRNA^{Ile}$	100-120†	R(-)	GCTCTAACCAGCTGAGCTAT
SP2F	16S-23S spacer	281-300†	F(+)	AAAACGAGATAAGTAAGTAA
SP2R	16S-23S spacer	281-300†	R(-)	GTGGATGCCTTGGCACTAG
SP3F	16S-23S spacer	390-410†	F(+)	CACTCACAAGATTAATAACG
SP3R	16S-23S spacer	390-410†	R(-)	CGTTATTAATCTTGTGAGTG
LR20R	23S	24-42*	F(+)	GTGGATGCCTTGGCACTAG
LR20F	23S	24-42*	R(-)	CTAGTGCCAAGGCATCCAC
LR194F (B)	23S	194-214*	R(-)	CTTTCTCTTCTCCGGGTACT‖
LR488 (C)	23S	502-520*	R(-)	CCTTTCCCTCACGGTACTG
LR488H‡	23S	502-520*	R(-)	AACCGGCCTTTCCCTCACGGTACTG
M13R	Phage M13	6205-6221§	F(+)	CAGGAAACAGCTATGAC
M13F	Phage M13	6291-6307§	R(-)	GTTTTCCCAGTCACGAC

Numbering according to;

*, the published 23S rRNA sequence from *S. aureus* (Ludwig *et al*, 1992);

†, the aligned spacer sequence for *S. aureus* (Fig. 3);

¶, the published 16S rRNA sequence from *S. aureus* (Ludwig *et al*, 1992);

§, the published sequence for bacteriophage M13;

‖, sequence obtained by the present inventor;

‡, addition of *Hpa* II sites (underlined) at 5' end of R1391F & LR488.

Letters in brackets indicate the designations of primers as given in Figure 1. As stated above, primers A and E are as disclosed in WO 93/11264.

Using the DNA typing approach described above and the primers LR1391F, LR1392F and LR488 as shown in Figure 9, PCR product C was amplified from the *S. aureus* strains listed in Table 3. These included 281 MRSA from four geographically distinct clinical sources and 48 penicillin or methicillin sensitive strains from a single Melbourne source; several methicillin-resistant or sensitive strains from type culture collections were also used. These strains yielded various amplified products, of which only the most intense bands were considered to be alleles. In total, 15 alleles, designated *rrnA* to *rrnO*, were recognised, with 14 varying in length from 935 to 1223 bp, as shown in Figure 10. *rrnO* was 906 bp in length (results not shown).

From Figure 10A, it can be seen that among the strains, two ribotypes, A and B, that were highly reproducible in individual isolates were obtained, with 104 and 174 strains respectively (including 5 type strains). An additional 7 ribotypes were found among the remaining 9 MRSA strains; Figure 10b shows 8 MRSA ribotypes. Ribotype A was the major ribotype found between 1960 and 1989 in Melbourne (19/22 strains), Singapore (7/9 strains), Ireland (9/9 strains), New York (1/1 strain) and UK (12/12

strains). After 1989, ribotype B was the major ribotype found at the Heidelberg Repatriation Hospital; 176 were ribotype B and 57 were ribotype A.

In contrast to the MRSA strains, the sensitive strains showed considerably more variation in the presence or absence of bands, yielding an additional 26 ribotypes from the 48 strains studied. Figure 10c shows some of these strains. The MRSA ribotypes A, B and I included some of the penicillin or methicillin sensitive strains. The occurrence of the alleles in the various ribotype classes is summarized in Table 8.

PCR product C was amplified from various *S. aureus* strains and separated by denaturing PAGE (Figure 10). The presence of variable length alleles (*rrn*A-O) is shown. The size of each allele is shown in Figure 10. The data were collated (using BioImage Software) from Figure 10 and four other denaturing polyacrylamide gels.

Table 8

Distribution of alleles in *S. aureus* ribotypes

Ribotype	Allele (*rrn*) A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	No. of Isolates
Methicillin sensitive																
Ma	+	-	+	-	-	-	+	-	+	-	-	-	-	-	+	2
Mb	-	-	+	-	-	-	-	+	-	+	-	+	-	-	-	1
Mc	-	-	+	-	-	+	+	+	+	-	-	-	-	-	-	1
Md	+	-	-	-	-	+	-	-	-	+	+	+	-	-	-	1
Me	-	-	+	+	+	+	-	+	-	+	+	-	-	-	-	1
Mf	-	-	+	+	+	+	-	+	-	-	-	-	-	-	-	1
Mg	-	-	+	+	+	-	+	+	-	-	-	-	-	-	-	2
Mh	-	+	+	+	+	+	-	+	-	+	+	-	-	-	-	2
Mi	+	-	+	+	-	-	+	+	-	-	-	-	-	-	-	2
Mj	-	-	+	-	-	+	+	+	+	+	+	+	-	-	-	2
Mk	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	2
Ml	+	-	-	-	-	+	-	-	-	+	-	+	-	-	-	1
Mm	-	-	+	-	+	+	-	+	-	-	-	-	-	-	-	1
Mn	+	+	-	-	-	+	-	-	+	+	-	+	-	-	-	1
Mo	+	-	+	-	-	+	-	-	+	+	-	+	-	-	-	1
Mp	+	-	+	-	-	-	-	+	-	-	-	+	-	-	-	2
Mq	+	-	+	+	-	+	+	-	+	-	-	-	-	-	-	1
Penicillin Sensitive																
Pa	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	2
Pc	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	6
Pd	+	-	+	+	+	-	+	+	-	+	-	-	-	-	-	1
Pe	-	-	+	-	-	+	-	-	-	+	-	+	-	-	-	1
Pf	-	-	+	-	-	+	-	+	-	+	-	+	-	-	-	1
Pg	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	1
Ph	-	-	+	-	-	+	+	+	-	+	-	+	-	-	-	1
Pi	+	-	-	-	+	-	+	+	+	-	-	-	-	-	-	2
Pj	+	-	+	-	-	-	+	-	+	-	-	-	-	-	-	1
MRSA																
A†	+	-	+	-	-	+	-	+	-	+	-	+	-	-	-	94
B§	+	-	+	-	-	+	-	+	-	+	-	-	-	-		178
C*	-	-	+	-	-	+	-	+	-	+	-	-	-	-	-	3
D‡	+	-	+	-	-	+	-	+	-	+	-	+	+	-		1
E	+	-	+	-	-	-	+			-	-	-	-	-		1
F	-	-	-	+	-	-		-	+	+	+	-	-	-		1
G	+	-	+	-	-	-	-	+	-	+	-	-	-	-	-	1
H	+	-	+	-	-	+	-	+	-	+	-	+	-	+	-	1
I¶	+	-	+	-	-	-	+	-	+	-	-	-	-	-	-	6

† including H11; one isolate was penicillin-sensitive and another was methicillin-sensitive;

§ including D46 and H12; one isolate was methicillin-sensitive;

¶ five isolates were methicillin-sensitive;

* including H14;

‡ including H21.

Example 7 Stability of Ribotypes of *Staphylococcus aureus*

The stability of ribotypes A, B, C, D and Pa was investigated by 30 serial passages of strains 9144, H11, H12, H14 and H21 over a six-week period. The ribotype was assessed after every fifth passage by visual comparison with reference patterns, and was found to be stable except for strains H12 and H21. Strain H12 was identified as ribotype B at all passages except the fifth, where *rrnL* appeared, making it ribotype A. Strain H21 was originally found to be ribotype D; however during the stability experiment it was found to be ribotype A at all passages subsequently investigated (the colonies from which the DNA prepared were used completely). After plating out colonies from the original frozen stock, genomic DNA was prepared from 10 separate colonies of strain H21: in all cases the ribotype was found to be A. The instability of strains H12 and H21 could be explained by a contaminant or by the rearrangement, duplication or deletion of an *rrn* operon to yield *rrnL* or *rrnM* respectively. This instability did not affect the typing of *S. aureus* strains significantly, since it was an infrequent event.

The instability of *rrn* operons has been reported in *B. subtilis* (Widom *et al*, 1988) and *E. coli* (Hill & Harnish, 1982). These reports show evidence for the loss of an *rrn* operon, and there is also evidence for recombination leading to chromosomal rearrangement of *rrn* operons (Hill & Harnish, 1982). The instability of ribotype D (strain H21) could be explained by the loss of

rrnM giving rise to the stable ribotype A. The relative instability of ribotype B in the present study was due to the loss of *rrnL*. The frequency of such events is low [~10^{-4}; (Hill & Harnish, 1982)] and thus will have little practical effect on this method.

<u>Example 8</u> <u>Sequencing of Variable Length 16S-23S rRNA Alleles</u>

In order to isolate and compare the variable length sequences of the 16S-23S rRNA alleles, PCR product C from strains D46 (ribotype B), H11 and ATCC33952 (both ribotype A) was cleaved with *Hpa*II and the resulting fragments cloned into M13 vectors. PCR product C (Figure 1) was amplified as described above from *S. aureus* genomic DNA from strains D46, H11, and 33952 using primers R1391F and LR488, R1391FH and LR488H or R1391F and LR194F (Table 7) . For each strain, ten equivalent reactions were pooled, precipitated with 26% w/v polyethylene glycol in 20mM $MgCl_2$ (Paithankar & Prasad, 1991) and digested with *Hpa*II. For D46, 1 g of the *Hpa*II digested products were end repaired with 4 units of T4 DNA polymerase (Boehringer), 200μM dNTPs, 33mM Tris-acetate (pH 8.0), 66mM potassium acetate, 10mM magnesium acetate, 0.5 mM dithiothreitol, 0.1mg bovine serum albumin ml^{-1} and incubated at 11°C for 30 minutes. For 33952 and H11 the *Hpa*II digested products (1-25ng) were ligated directly into *Acc*I digested M13mp19RF (50ng: in a total of 10μl) and the end repaired *Hpa*II digested products (1-25ng) from strain D46 were ligated into *Sma*I digested M13mp19RF (50ng: in a total of 10 μl) with 1 unit T4 DNA ligase (Boehringer), 66mM Tris-HCl (pH 7.5), 5mM $MgCl_2$, 1mM dithiothreitol, 1mM ATP and incubated at room temperature overnight. The competent JM109 *E. coli* cells (50μl; Promega) were transformed with 2-3μl of the ligation mixtures according to the protocol described in Sambrook *et al* (1989). After plates were incubated overnight at 37°C, bacteriophage M13 plaques were either picked off and grown in Luria Broth

(LB) or colony hybridizations (Sambrook *et al*, 1989) to *Hpa*II digested PCR product C labelled with digoxigenin were performed. Positive plaques were then picked off and grown in LB (Sambrook *et al*, 1989). Single stranded bacteriophage M13 DNA was then prepared from all the positive clones (Sambrook *et al*, 1989). To determine the presence and size of the inserts, the single stranded DNA from the M13 clones was used as a template in the PCR using M13F and R primers which flank the *Sma*I and *Acc*I restriction sites (Yanisch-Perron *et al*, 1985). Four clones were isolated from D46, one containing insert E, one containing insert F, one containing insert G and one containing insert H. Sixteen clones, designated V2-V17 were isolated from H11; seven contained insert E, four contained insert H, four contained insert G and one contained insert F. With the sequence information of insert (H), primer LR194F was designed so as to contain a *Hpa*II site. For strain 33952, primers LR194F and R1391F were used to obtain a mixture of PCR products, which were digested with *Hpa*II to yield predominantly product E and cloned into M13mp19; clones V18-V47 were isolated, and of these 9 contained insert E. These results are summarized in Table 9.

Table 9

The presence or absence of sequences within the nine (rrnA,C, E,F,G,H,J,K & L) 16S-23S spacer alleles sequenced

Allele	Clone	Strain isolated	GenBank no.§	bp*	bp‡	bp¶	CS1	tRNA Ile	VS1	VS2	tRNA ala	VS3	VS4	CS2	VS5	VS6	VS7	CS3	VS8	Base pair changes
							Position													
							1-73	93-175	74-119	120-228	176-270	232-270	248-270	271-415	416-449	450-527	530-537	538-546	547-550	
rrnL	V13	H11	U11775	303	603	981	+	-	+	-	-	-	+	+	-	-	-	+	-	na
rrnK	V41	33952	U11785	319	616	994	+	-	+	-	-	+	-	+	-	-	-	+	-	na
rrnJ	4.	D46	U11773																	
	V4.	H11	U11783																	
	V17.	H11	U11776	335	634	1012	+	+	-	-	-	-	-	+	-	-	-	+	-	5
	V8.	H11	U11789																	
	V32.	33952	U11780																	
	V43	33952	U11787																	
rrnH	V27	33952	U11778	362	661	1039	+	-	+	-	-	+	-	+	+	-	+	+	+	2
	V7	H11	U11788		663	1041														
rrnG	V2	H11	U11777	382	672	1050	+	+	-	-	-	-	-	+	+	-	+	+	+	na
rrnF	V30	33952	U11779	460	756	1134	+	+	-	-	-	-	-	+	+	+	+	+	+	4
	V38	33952	U11782																	
rrnE	V12	H11	U11774	469	764	1142	+	-	+	+	-	+	-	+	+	-	+	+	+	na
rrnC	V34	33952	U11781	473	774	1152	+	+	-	-	+	-	-	+	+	-	+	+	+	5
	V42	33952	U11786																	
rrnA	V40	33952	U11784	551	849	1227	+	+	-	-	+	-	-	+	+	+	+	+	+	na
Length							73	82	33	109	94	38	23	145	33	77	8	8	4	

It was confirmed that the inserts were of variable length when amplified by PCR and then digested with *Dra*I or *Hinf*I (results not shown; restriction enzyme cleavage sites are indicated in Figure 1). It was determined from the first insert sequenced (clone 4) that *Hinf*I digested at the and of the $tRNA^{ile}$ gene (Figures 1, 11b). All the PCR products which were digested with *Hinf*I contained a $tRNA^{ile}$ gene, and this was subsequently confirmed by DNA sequencing, as shown in Figure 11b.

The 16S-23S rDNA spacer sequences of 9 *rrn* operons were determined from 3 methicillin resistant *S. aureus* strains. The variation in 16S-23S spacer length (303 bp to 551 bp) was accounted for by the type ($tRNA^{ala}$ or $tRNA^{ile}$) and number (one, both or none) of tRNA genes, and by the presence or absence of other sequences of unknown function.

<u>Example 9</u> <u>Designation of Alleles</u>

The designation of alleles set out in Table 9 was made by direct correlation with fragment C molecular weights (Figure 10A). The length of the spacer varied from 303 bp to 551 bp. The fragment E insert sequences were aligned to the 16S, 23S and 16S-23S spacer rDNA sequences, as shown in Figures 11a, 11c and 11b respectively. There were only 4 base pair differences in the 16S rDNA sequences, in contrast to 71 base pair differences in the 23S rDNA sequences. In the 16S-23S spacer rDNA (Figure 11b and Table 9) there were no differences in CS1 and CS2; however, there were striking gaps between alleles in regions VS2, $tRNA^{ala}$, VS3, VS4, VS5, VS6, VS7, and VS8. The $tRNA^{ile}$ gene was present in *rrn*J,G,F,C and A, while the $tRNA^{ala}$ gene was only present in *rrn*A and C. The number of base pair differences between clones judged to be the same allele was 5 for *rrn*J (from strains H11, D46 and 33952, isolated in 1982, 1992 and 1981 respectively), 2 for *rrn*H (from strains 33952 and H11), 4 for *rrn*F (strain 33952) and 5 for *rrn*C (33952).

Using phylogenetic analysis, the *rrn* alleles were divided into 3 distinct groups, which are shown in Figure 12; "a" contains $tRNA^{ile}$ and $tRNA^{ala}$, "b" contains $tRNA^{ile}$ only and "c" contains no tRNA genes.

To confirm the presence of variable length 16S-23S spacer regions in genomic DNA, PCR products I and J were hybridized to genomic DNA isolated from *S. aureus* isolates. Between 4 and 7 bands ranging from ~600 bp to ~850 bp were obtained for all strains, with variation between strains. The results are in close agreement with the results obtained with PCR-ribotyping (Figure 10) and DNA sequencing (Figure 11 and Table 9).

The type of tRNA gene found in the 16S-23S spacer varies in number (0, 1 or 2) and combination between operon and between species; *A. hydrophila* (East & Collins, 1993) and *E. coli* (Morgan *et al*, 1977) have $tRNA^{ala}$, $tRNA^{ile}$ and $tRNA^{glu}$; *B. subtilis* (Loughney *et al*, 1982) and *C. crescentus* (Feingold *et al*, 1985) have $tRNA^{ile}$ and $tRNA^{ala}$; *P. shigelloides* (East *et al*, 1992) has $tRNA^{glu}$; *Methanococcus vanielli* (Jarsch & Böck, 1983) and *Enterococcus hirae* (Sechi & Daneo-Moore, 1993) have $tRNA^{ala}$; and *Mycobacterium bovis* has no tRNA genes (Suzuki *et al*, 1987). The length of the spacer varies from 156 bp for *M. vanielli* (Jarsch & Böck, 1983) to 551 bp (present specification). We have now found that there is intraspacer and interspacer variation of other sequences besides tRNA genes.

Our results show that variable length 16S-23S spacer regions occur in genomic DNA whose size range is similar to the results obtained with PCR-ribotyping (Figure 10) and DNA sequencing (Table 9). The majority of strains presented in this study were from the Heidelberg Repatriation Hospital (274 strains of a total of 322). Among the MRSA strains, two ribotypes (A and B) that were highly reproducible in individual isolates were obtained (Figure 10a), with 101 and 180 strains respectively (including 5 type strains). An additional 7 ribotypes were

found among the remaining 9 MRSA strains (Figure 10b shows 8 MRSA ribotypes). Ribotype A was the major ribotype found (ribotype A/total no of strains in location) between 1960 and 1989 in Melbourne (19/22), Ireland (9/9), New York (1/1) and UK (12/12). After 1989, ribotype B was the major ribotype found at the HRH (176 were ribotype B and 57 were ribotype A). In contrast to the MRSA strains, the sensitive strains showed considerably more variation in the presence or absence of bands, yielding an additional 26 ribotypes from the 48 strains studied (Figure 10c shows some of these strains). The MRSA ribotypes A, B and I included some of the penicillin or methicillin sensitive strains. The occurrence of the alleles in the various ribotype classes is summarized in Table 7.

Thus we have shown that the presence or absence of specific variable length rDNA spacer regions varies between *S. aureus* strains. The patterns obtained were mostly stable within strains upon repeated testing, allowed the designation of strains to specific types, discriminated within the species *S. aureus*, and allowed for the easy testing of large numbers of strains. With these criteria met, the molecular typing method described here is useful for epidemiological studies of *S. aureus*.

The variation in length and sequence of the 16S-23S spacer makes it an ideal candidate for typing of strains and species identification which can potentially be applied to any species of the bacterial kingdom. Our method permits reliable, rapid identification and typing on this basis.

The sequence information presented in Figure 11 is tabulated to show from which *S. aureus* strains the original PCR product was isolated, which clones were characterized, the size of the 16S-23S spacer (*), the size of the *Hpa*II insert (fragment E: ‡), the presence or absence of sequences, with positions and lengths shown according to numbering in Figure 13), the base pair differences between clones for the 13 regions (CS=conserved

sequence, VS=variable sequence) and the GenBank accession numbers §. The size (base pairs, bp) of fragment C (¶) can be obtained by adding 331bp (*Hpa*II^2 to LR520) and 47bp (R1392 to *Hpa*II^1) to fragment E (‡).

Whereas the bacteriophage typing system was formerly the standard method for *S. aureus* (Williams *et al*, 1953), many current MRSA strains are not typable by the International Set of Phages (Richardson *et al*, 1988), requiring the addition of further experimental phages. RFLP analysis by pulsed-field-gel electrophoresis has been shown to be more discriminating than phage typing (Schlichting *et al*, 1993). However, RFLP analysis relies on the stability of restriction enzyme recognition sites such that a point mutation within a site will result in a different RFLP. The sequence conservation of the *rrn* operons (Woese, 1987) argues for the use of the 16S-23S spacer region as a more stable and direct indicator of the evolutionary divergence of *S. aureus* strains, and is a valuable addition to the large number of typing methods available.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

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CLAIMS:

1. A method of identification of microorganisms, comprising the steps of extracting and purifying DNA from a sample suspected to contain bacteria, and subjecting the 16S-23S rRNA spacer region of said DNA to amplification, comprising a highly conserved region from the 3' end of the 16S-23S rRNA spacer region, and/or a highly conserved region from the 5' end of said region. thereby producing fragments having detectable differences in size and number, and separating the amplified fragments.

2. A method according to Claim 1, wherein the primers used correspond to a highly conserved region from the 3' end of the spacer region, and to a highly conserved region from the 5' end of the spacer region respectively.

3. A method according to Claim 1 or Claims 2, wherein the primers comprise a sequence corresponding to a region from the 5' end of the 16S rRNA gene and/or to a region from the 3' end of the 23S rRNA gene.

4. A method according to any one of Claims 1 to 3, using a first primer comprising a sequence from the 5' end of the 16S rRNA gene, and a second primer comprising a sequence from the 3' end of the 23S rRNA gene.

5. A method according to any one of Claims 1 to 4, wherein the primers are 15 to 20 nucleotides long.

6. A method according to Claim 5, wherein the primers are respectively R1391F and selected from the group consisting of LR488 and LR194F, said primers being as herein defined.

7. A method according to Claim 6, wherein LR488 is 15 to 19 nucleotides long, and R1391F is 15 to 18 nucleotides long.

8. A method according to any one of Claims 1 to 7, wherein one or more additional probes are used.

9. A method according to Claim 8, wherein the additional probe is the sequence encoding $tRNA^{ile}$ and/or the sequence encoding $tRNA^{ala}$.

10. A method according to any one of Claims 1 to 9, wherein the amplification is performed by a method selected from the group consisting of polymerase chain reaction, ligase chain reaction, 3SR amplification, strand displacement amplification, Qβ replicase reaction, and branched DNA signal amplification.

11. A method according to any one of Claims 1 to 9, wherein the amplified fragments are separated by a method selected from the group consisting of denaturing polyacrylamide gel electrophoresis, capillary electrophoresis, and high performance liquid chromatography.

12. A method according to any one of Claims 1 to 11, wherein the sample is a clinical or environmental sample.

13. A method according to any one of Claims 1 to 11, wherein the microorganism is a *Clostridium* or a *Staphylococcus*.

14. An amplification primer reagent for use in a method according to any one of Claims 1 to 13, comprising a highly conserved region from the 3' end of the 16S-23S rRNA spacer region, and/or a highly conserved region from the 5' end of said region.

15. An amplification primer reagent according to Claim 14, comprising a sequence corresponding to a region from the 5' end of the 16S rRNA gene and/or to a region from the 3' end of the 23S rRNA gene.

16. An amplification primer reagent according to Claim 15, comprising a region from the 5' end of the 16S rRNA gene and a region from the 3' end of the 23S rRNA gene.

17. An amplification primer reagent according to any one of Claims 14 to 17, wherein the primers are 15 to 20 nucleotides long.

18. An amplification primer reagent according to Claim 16, wherein the primers are respectively R1391F and selcted from the group consisting of LR488 and LR194F, said primers being as herein defined.

19. An amplification primer reagent according to Claim 18, wherein LR488 is 15 to 19 nucleotides long, and R1391F is 15 to 18 nucleotides long.

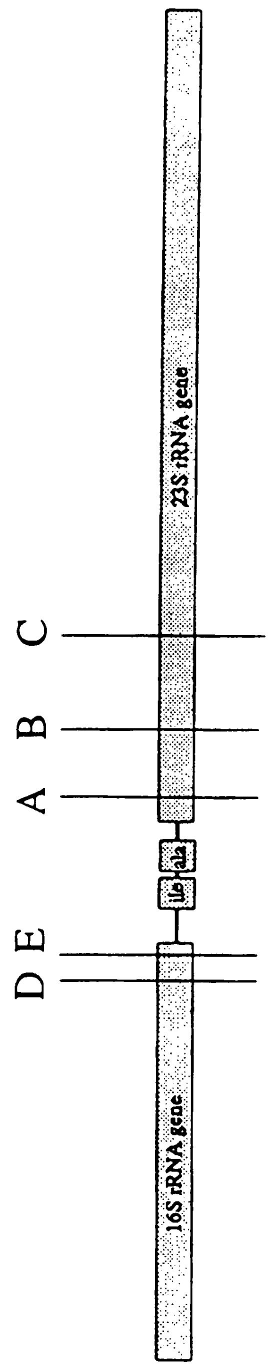

FIGURE 1

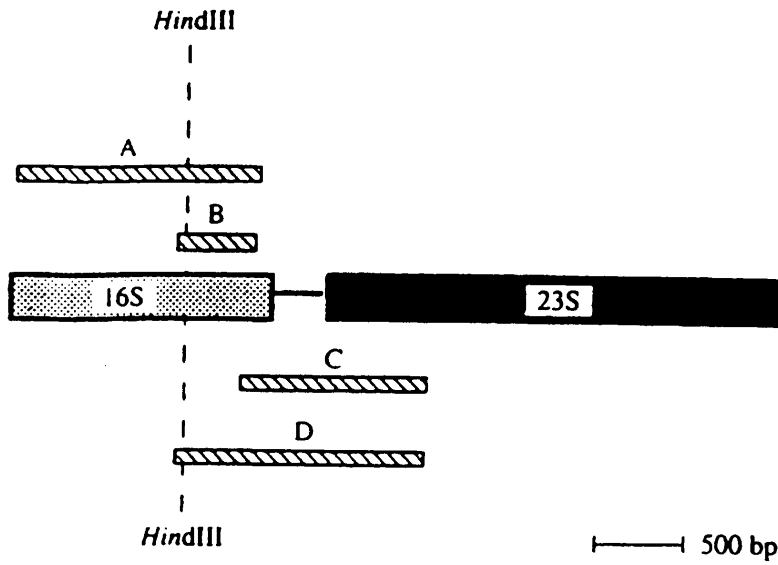

FIGURE 2

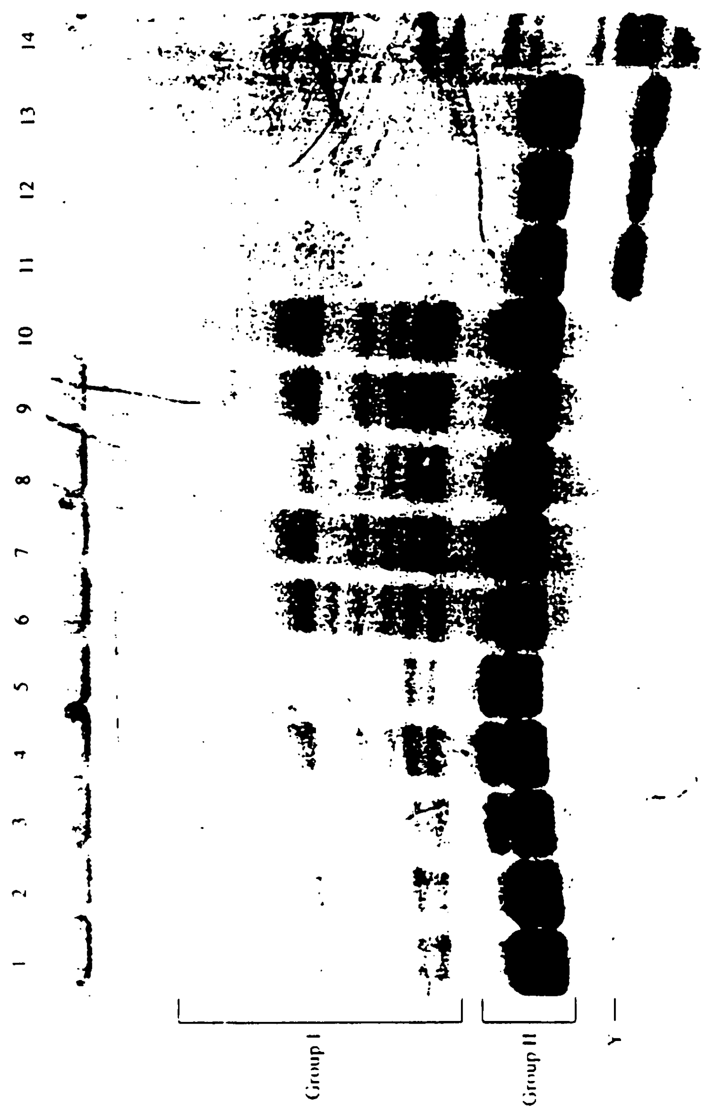

FIGURE 3

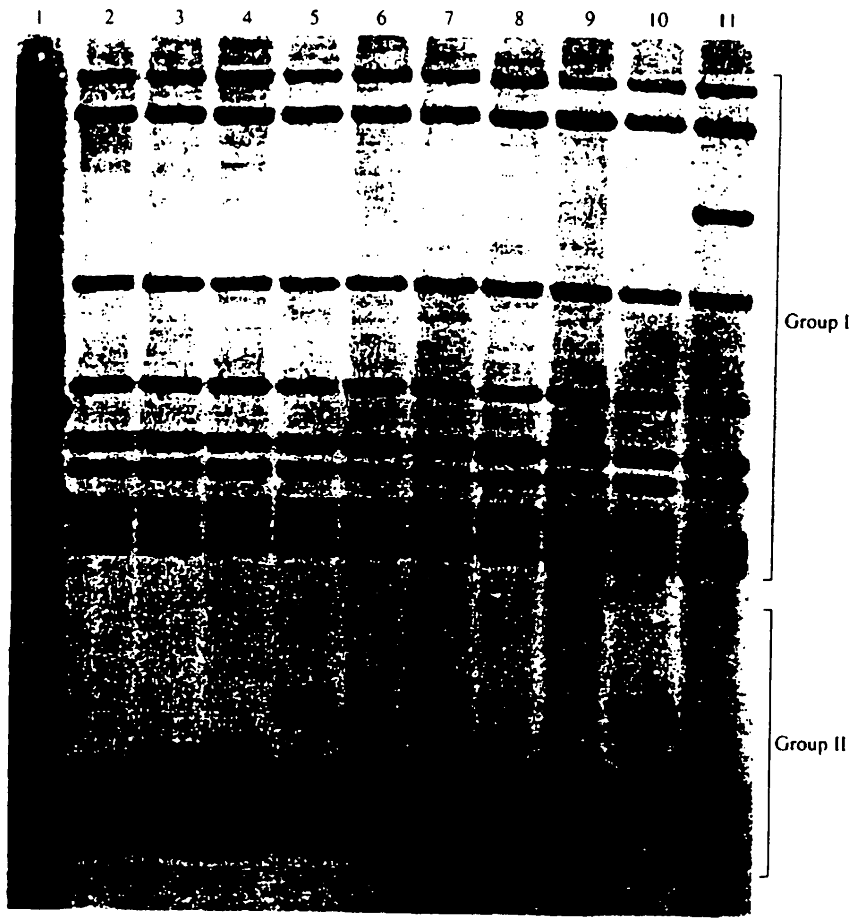

FIGURE 4

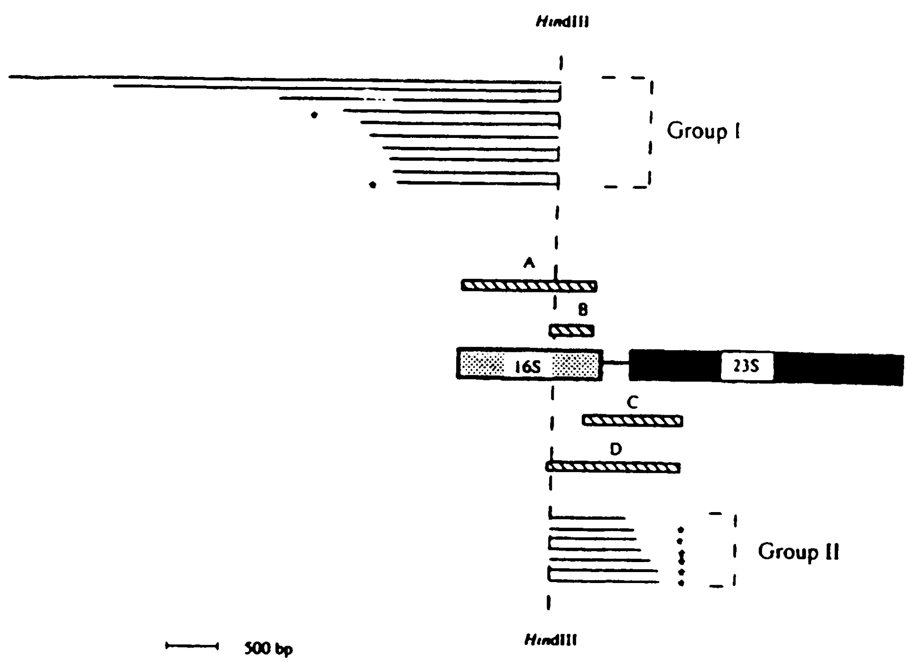

FIGURE 5

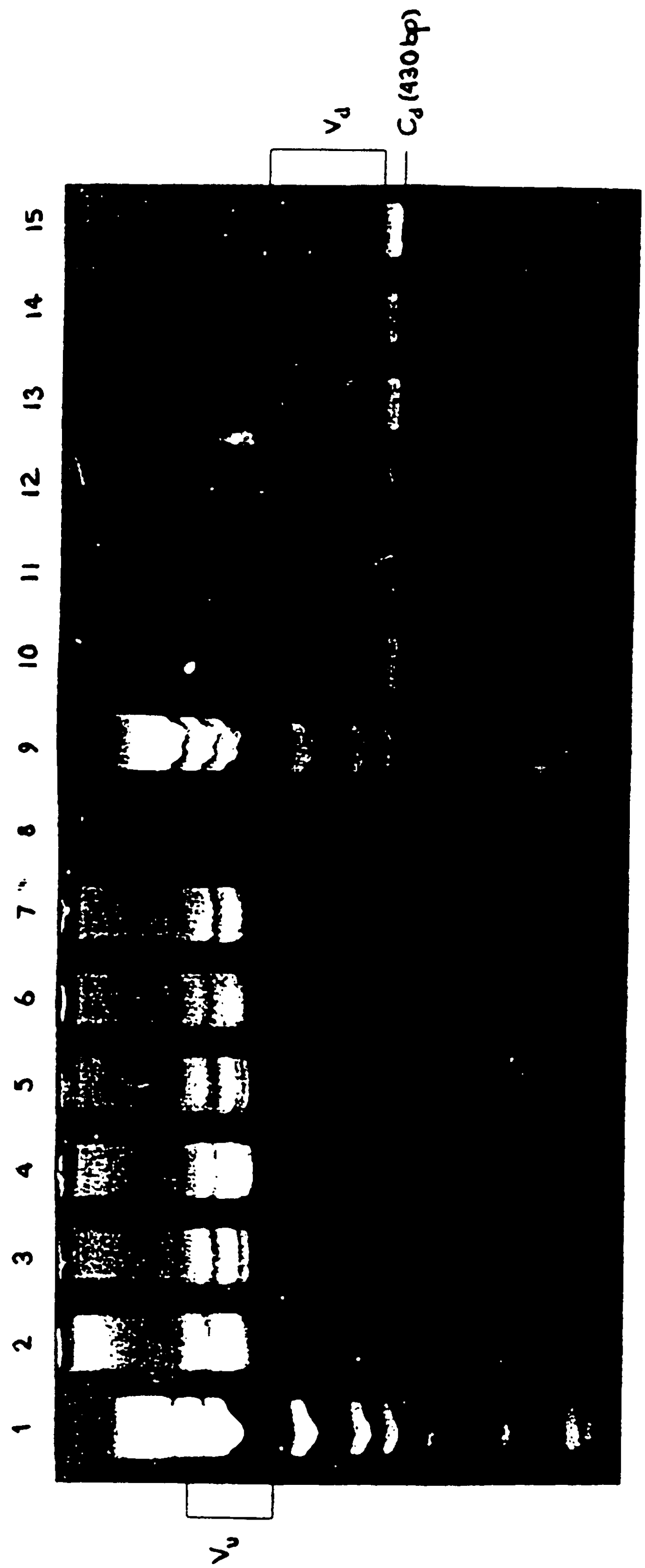

FIGURE 6

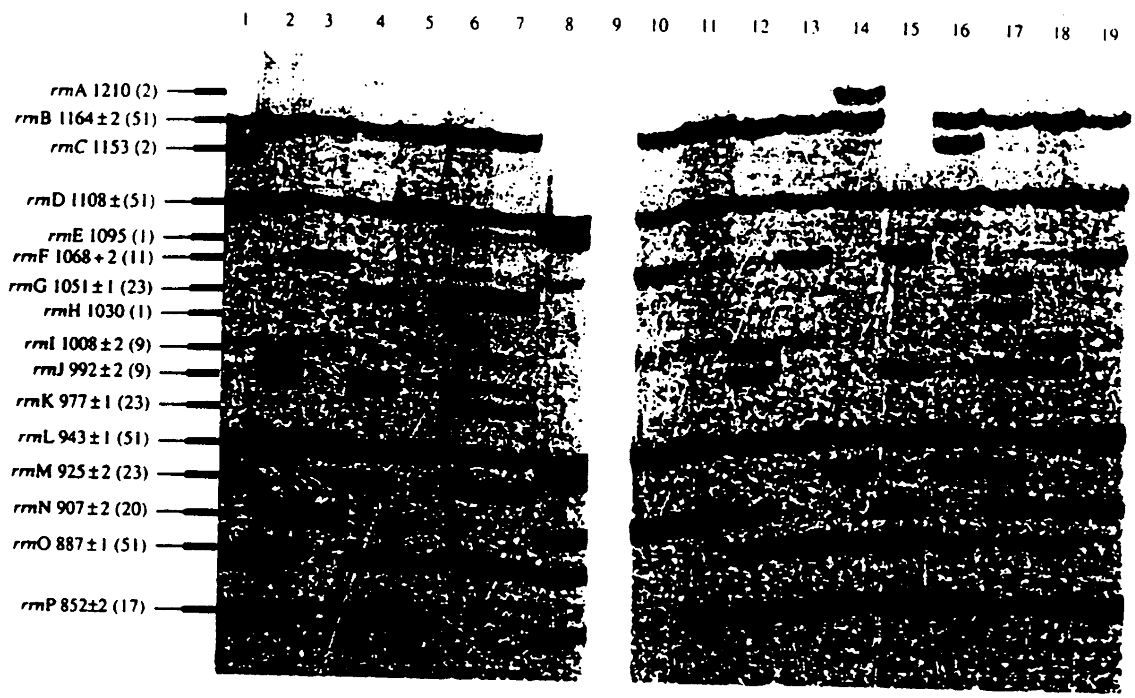
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
rrnA 1210 (2)
rrnB 1164±2 (51)
rrnC 1153 (2)
rrnD 1108±(51)
rrnE 1095 (1)
rrnF 1068+2 (11)
rrnG 1051±1 (23)
rrnH 1030 (1)
rrnI 1008±2 (9)
rrnJ 992±2 (9)
rrnK 977±1 (23)
rrnL 943±1 (51)
rrnM 925±2 (23)
rrnN 907±2 (20)
rrnO 887±1 (51)
rrnP 852±2 (17)

FIGURE 7

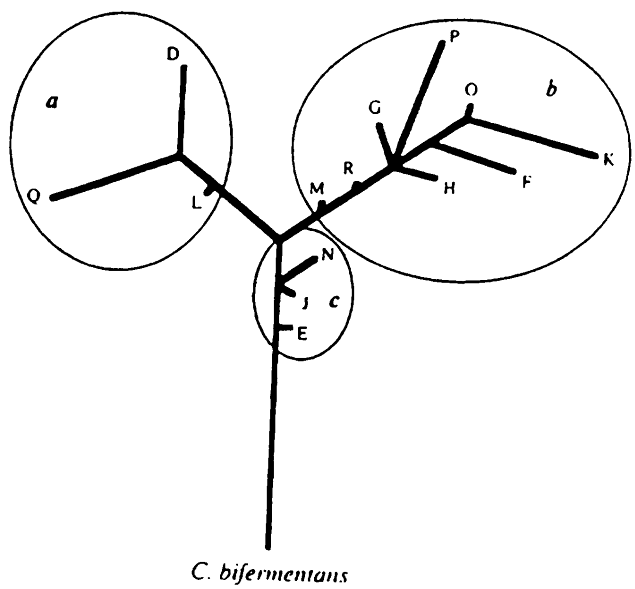

FIGURE 8

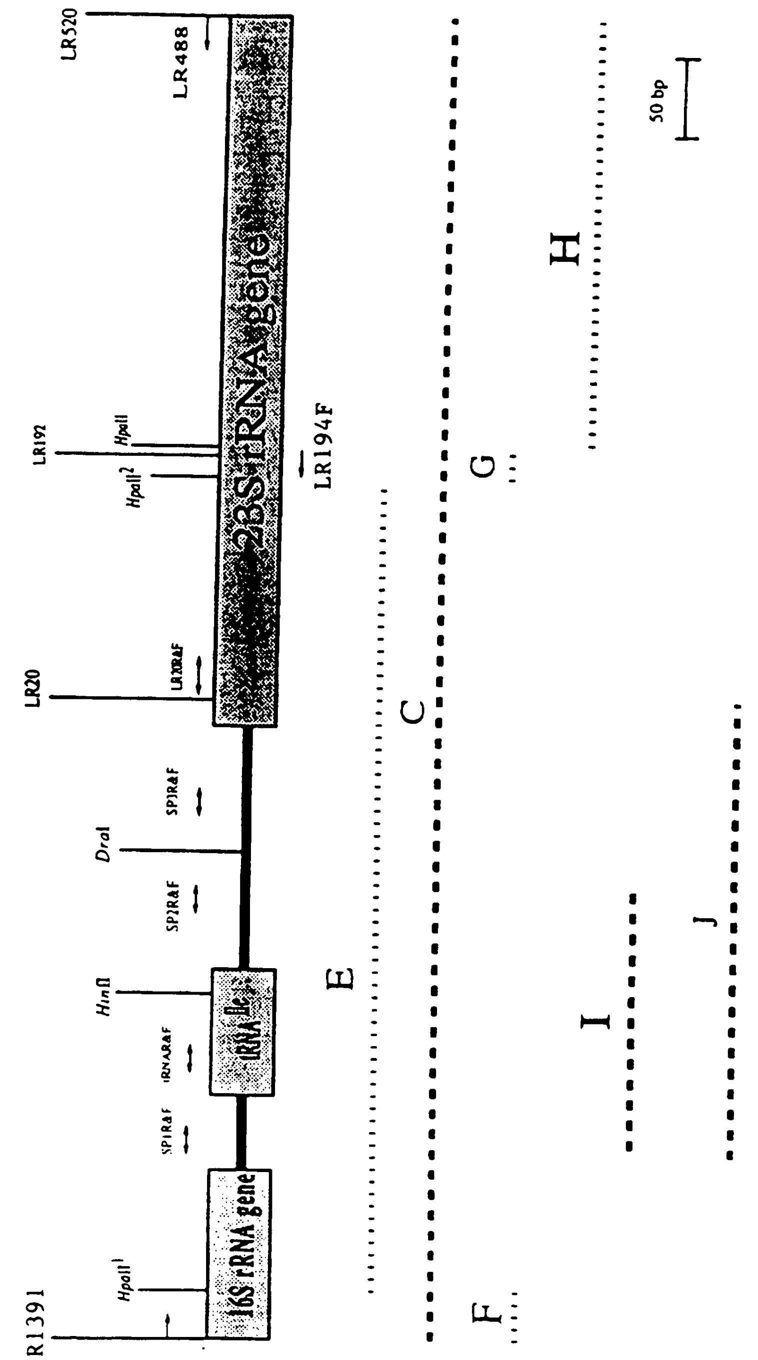
R1391
HpaII¹
16S rRNA gene
SP1RAF
tRNARAF
tRNA Ile
HinfI
SP2RAF
DraI
SP3RAF
LR20
LR20RAF
23S rRNA gene
HpaII²
LR192
HpaII
LR194F
LR488
LR520
50 bp
E
C
F
G
H
I
J

FIGURE 9

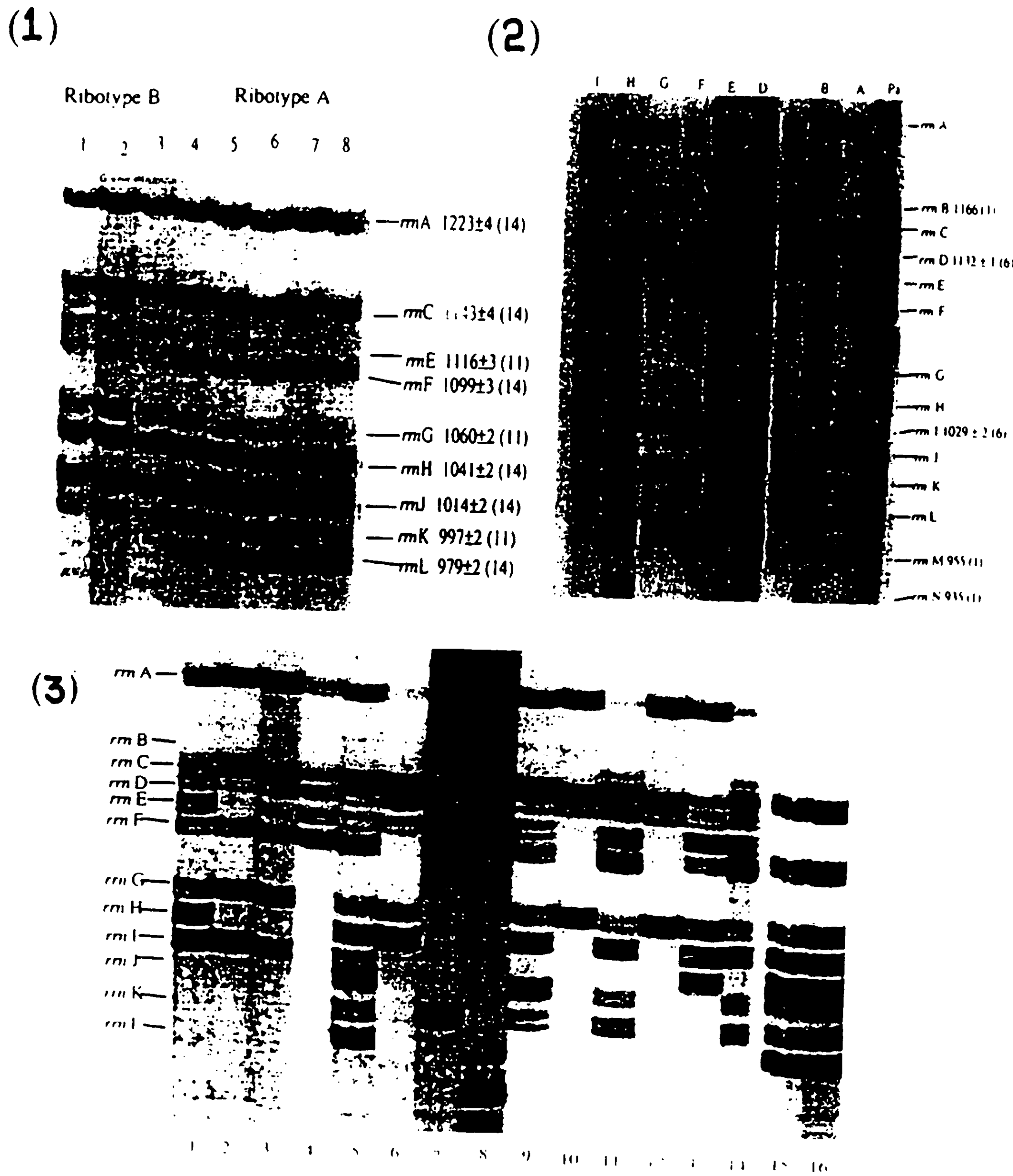

FIGURE 10

SUBSTITUTE SHEET (RULE 26)

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                1456        1466        1476        1486        1496        1506        1516        1526
SA16S     CGGTGGAGTA  ACCTTTTAGG  AGCTAGCCGT  CGAAGGTGGG  ACAAATGATT  GGGGTGAA-G  TCGTAACAAG  GTAGCCGTAT
rrnG      ..........  ..........  ..........  ..........  ..........  ........-.  ..........  ..........
rrnC  V34 ..........  ..........  C.........  ..........  ..........  ........-.  ..........  ..........
rrnC  V42 ..........  ..........  ..........  ..........  ..........  ........-.  ..........  ..........
rrnI      ..........  ..........  ..........  ..........  ..........  ........-.  ..........  ..........
rrnH  v7  ..........  ..........  ..........  ..........  ..........  ........-.  ..........  ..........
rrnH  V27 ..........  ..........  ..........  ..........  ..........  ........T.  ..........  ..........
rrnK      ..........  ..........  .......-..  ..........  ..........  ........-.  ..........  ..........

                                1536        1546        1556
SA16S                     CGGAAGGTGC  GGCTGGATCA  CCTCCTTTCT
rrnJ,F,C,A,H,K,L          ..........  ..........  ..........
rrnE                      ........-.  ..........  ..........
```

FIGURE 11a

```
               10         20         30         40         50         60         70         80
rrn J  AAGGATATAT TCGGAACATC TTCTTCAGAA GATGCGGAAT AACGTGACAT ATTGTATTCA GTTTTGAATG TTTATTTAAC
rrn G  AAGGATATAT TCGGAACATC TTCTTCAGAA GATGCGGAAT AACGTGACAT ATTGTATTCA GTTTTGAATG TTTATTTAAC
rrn C  AAGGATATAT TCGGAACATC TTCTTCAGAA GATGCGGAAT AACGTGACAT ATTGTATTCA GTTTTGAATG TTTATTTAAC
rrn A  AAGGATATAT TCGGAACATC TTCTTCAGAA GATGCGGAAT AACGTGACAT ATTGTATTCA GTTTTGAATG TTTGTT---C
rrn F  AAGGATATAT TCGGAACATC TTCTTCAGAA GATGCGGAAT AACGTGACAT ATTGTATTCA GTTTTGAATG TTTGTT---C
rrn E  AAGGATATAT TCGGAACATC TTCCTCAGAA GATGCGGAAT AACGTGACAT ATTGTATTCA GTTTTGAATG TTTATTTAAC
rrn H  AAGGATATAT TCGGAACATC TTCCTCAGAA GATGCGGAAT AACGTGACAT ATTGTATTCA GTTTTGAATG TTTATTTAAC
rrn K  AAGGATATAT TCGGAACATC TTCTTCAGAA GATGCGGAAT AATGTGACAT ATTGTATTCA GTTTTGAATG TTTATTTAAC
rrn L  AAGGATATAT TCGGAACATC TTCTTCAGAA GATGCGGAAT AATGTGACAT ATTGTATTCA GTTTTGAATG TTTATTTAAC

               90        100        110        120        130        140        150        160
rrn J  ATTCAAAAAA ATGG-GCCTA TA-GCTCAGC TGGTTAGAGC GCACGCCTGA TAAGCGTGAG GTCGGTGGTT CGAGTCCAC-
rrn G  ATTCAAA-AA ATGG-GCCTA TA-GCTCAGC TGGTTAGAGC GCACGCCTGA TAAGCGTGAG GTCGGTGGGT CGAGTCCAC-
rrn C  ATTCAAATTA ATGG-GCCTA TA-GCTCAGC TGGTTAGAGC GCACGCCTGA TAAGCGTGAG GTCGGTGGTT CGAGTCCAC-
rrn A  ATTCAAATTA ATGG-GCCTA TA-GCTCAGC TGGTTAGAGC GCACGCCTGA TAAGCGTGAG GTCGGTGGTT CGAGTCCAC-
rrn F  ATTCAAAAAA ATGG-GCCTA TA-GCTCAGC TGGTTAGAGC GCACGCCTGA TAAGCGTGAG GTCGGTGGTT CGAGTCCAC-
rrn E  ATTCAAATAT TTTTTGGTTA AA-GTGATAT TGCTTAT-GC GAGCGCTTGA CAATC-TATT CTTTTTAAAG AAAGCGGTTG
rrn H  ATTCAAATAT TTTTTGGTTA AA-GTGATAT TGCTTAT-G- ---------- ---------- ---------- ----------
rrn K  ATTCAAATAT TTTTTGGTTA AACGTGATAT TGCTTAT-G- ---------- ---------- ---------- ----------
rrn L  ATTCAAATAT TTTTTGGTTA AAG-TGATAT TGCTTAT-G- ---------- ---------- ---------- ----------

              170        180        190        200        210        220        230        240
rrn J  TTAGGCCCAC CATTA----- ---------- ---------- ---------- ---------- ---------- ----------
rrn G  TTAGGCCCAC CATTA----- ---------- ---------- ---------- ---------- ---------- ----------
rrn C  TTAGGCCCAC CATTAATTTA ATACCTATTT GGGGGCTTAG CTCAGCTGGG AGAGCGCCTG CTTTGCACGC AGGAGGTCAG
rrn A  TTAGGCCCAC CATTAATTTA ATACCTATTT GGGGGCTTAG CTCAGCTGGG AGAGCGCCTG CTTTGCACGC AGGAGGTCAG
rrn F  TTAGGCCCAC CATTA----- ---------- ---------- ---------- ---------- ---------- ----------
rrn E  TTAGACAATG CATTAA--GA AAAATTAAAG CGGAGTTTAC TTTTGTAAAT -GAGCATTTG ATTTTTTG-- -AAAATAAAG
rrn H  ---------- ---------- ---------- ---------- ---------- ---------- ---------- -AAAATAAAG
rrn K  ---------- ---------- ---------- ---------- ---------- ---------- ---------- -AAAATAAAG
rrn L  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

              250        260        270        280        290        300        310        320
rrn J  ---------- ---------- ---------T TTGTACATTG AAAACTAGAT AAGTAAGTAA AATATAGATT TTACCAAGCA
rrn G  ---------- ---------- ---------T TTGTACATTG AAAACTAGAT AAGTAAGTAA AATATAGATT TTACCAAGCA
rrn C  CGGTTCGATC CCGCTAGTCT CCACCATTAT TTGTACATTG AAAACTAGAT AAGTAAGTAA AATATAGATT TTACCAAGCA
rrn A  CGGTTCGATC CCGCTAGTCT CCACCATTAT TTGTACATTG AAAACTAGAT AAGTAAGTAA AATATAGATT TTACCAAGCA
rrn F  ---------- ---------- ---------T TTGTACATTG AAAACTAGAT AAGTAAGTAA AATATAGATT TTACCAAGCA
rrn E  CAGTATGCGA GCGCTTGACT AAAAAG-AAA TTGTACATTG AAAACTAGAT AAGTAAGTAA AATATAGATT TTACCAAGCA
rrn H  CAGTATGCGA GCGCTTGACT AAAAAG-AAA TTGTACATTG AAAACTAGAT AAGTAAGTAA AATATAGATT TTACCAAGCA
rrn K  CAGTATGCGA GCGCTTGACT AAAAAG-AAA TTGTACATTG AAAACTAGAT AAGTAAGTAA AATATAGATT TTACCAAGCA
rrn L  -------CGA GCGCTTGACT AAAAAG-AAA TTGTACATTG AAAACTAGAT AAGTAAGTAA AATATAGATT TTACCAAGCA

              330        340        350        360        370        380        390        400
rrn J  AAACCGAGTG AATAAAGAGT TTTAAATAAG CTTGAATTCA TAAGAAATAA TCGCTAGTGT TCGAAAGAAC ACTCACAAGA
rrn G  AAACCGAGTG AATAAAGAGT TTTAAATAAG CTTGAATTCA TAAGAAATAA TCGCTAGTGT TCGAAAGAAC ACTCACAAGA
rrn C  AAACCGAGTG AATAAAGAGT TTTAAATAAG CTTGAATTCA TAAGAAATAA TCGCTAGTGT TCGAAAGAAC ACTCACAAGA
rrn A  AAACCGAGTG AATAAAGAGT TTTAAATAAG CTTGAATTCA TAAGAAATAA TCGCTAGTGT TCGAAAGAAC ACTCACAAGA
rrn F  AAACCGAGTG AATAAAGAGT TTTAAATAAG CTTGAATTCA TAAGAAATAA TCGCTAGTGT TCGAAAGAAC ACTCACAAGA
rrn E  AAACCGAGTG AATAAAGAGT TTTAAATAAG CTTGAATTCA TAAGAAATAA TCGCTAGTGT TCGAAAGAAC ACTCACAAGA
rrn H  AAACCGAGTG AATAAAGAGT TTTAAATAAG CTTGAATTCA TAAGAAATAA TCGCTAGTGT TCGAAAGAAC ACTCACAAGA
rrn K  AAACCGAGTG AATAAAGAGT TTTAAATAAG CTTGAATTCA TAAGAAATAA TCGCTAGTGT TCGAAAGAAC ACTCACAAGA
rrn L  AAACCGAGTG AATAAAGAGT TTTAAATAAG CTTGAATTCA TAAGAAATAA TCGCTAGTGT TCGAAAGAAC ACTCACAAGA

              410        420        430        440        450        460        470        480
rrn J  TTAATAACGC GTTT------ ---------- ---------- ---------- ---------- ---------- ----------
rrn G  TTAATAACGC GTTTAAATCT TTTTATAAAA GAACGTAACT TCATGTTAA- ---------- ---------- ----------
rrn C  TTAATAACGC GTTTAAATCT TTTTATAAAA GAACGTAACT TCATGTTAA- ---------- ---------- ----------
rrn A  TTAATAACGC GTTTAAATCT TTTTATAAAA GAAAACGTTT AGCAGACAAT GA-GTTAAAT TATTTTAAAG CAGGAGTTTA
rrn F  TTAATAACGC GTTTAAATCT TTTTATAAAA GAAAACGTTT AGTAGACAAT GAAGTTAAAT TATTTTAAAG CAG-AGTTTA
rrn E  TTAATAACGC GTTTAAATCT TTTTATAAAA GAACGTAACT TCATGTTAA- ---------- ---------- ----------
rrn H  TTAATAACGC GTTTAAATCT TTTTA--AAA GAACGTAACT TCATGTTAA- ---------- ---------- ----------
rrn K  TTAATAACGC GTTT------ ---------- ---------- ---------- ---------- ---------- ----------
rrn L  TTAATAACGC GTTT------ ---------- ---------- ---------- ---------- ---------- ----------

              490        500        510        520        530        540        550        559
rrn J  ---------- ---------- ---------- ---------- ---------- ------CCTG TAGGA----T GGAAACATA
rrn G  ---------- ---------- ---------- ---------- --------CG TTTGGACTTA TAAAAATGGT GGAAACATA
rrn C  ---------- ---------- ---------- ---------- --------CG TTT-GACTTA TAAAAATGGT GGAAACATA
rrn A  CTTATGTAAA TGAGCATTTA AAATAATGAA AACGAAGCCG TATGTGAGCG TTT-GACTTA TAAAAATGGT GGAAACATA
rrn F  CTTATGTAAA TGAGCATTTA AAATAATGAA AACGAAGCCG TATGTGAGCG TTT-GACTTA TAAAAATGGT GGAAACATA
rrn E  ---------- ---------- ---------- ---------- --------CG TTT-GACTTA TAAAAATGGT GGAAACATA
rrn H  ---------- ---------- ---------- ---------- --------CG TTT-GACTTA TAAAAATGGT GGAAACATA
rrn K  ---------- ---------- ---------- ---------- ---------- ------CCTG TAGGA----T GGAAACATA
rrn L  ---------- ---------- ---------- ---------- ---------- ------CCTG TAGGA----T GGAAACATA
```

FIGURE 11b

```
SA23S        GATTAAGTTA TTAAGGGCGC ACGG--TGGA TGCCTTGGCA CTAGAAGCCG ATGAAGGACG TTACTAACGA CGATATGCTT
rrnJ‡        .......... .......... ....--.... .......... .......... .......... .......... ..........
rrnJ-V4      .......... .......... ..A.GA.... .......... .......... .......... .......... ..........
rrnJ-V8      .......--. .......... ....--.... .......... .......... ......-... .......... ..........
rrnJ-V43     .......... .......... ....--.... .......... .......... .....TT... .......... ..........
rrnE         .......... .........- ....--...- .......... .......... -......... .......... ---.......
rrnF         .......... .......... ....--.... .......... .......... .......... .......... ..........
rrnF         .......... .........- ....--.... .......... .......... .......... .......... ..........
rrnA,C,K,H   .......... .......... ....--.... .......... .......... .......... .......... ..........
rrnE         .......... .......... ..A.--.... .......... .......... .......... .......... ..........
rrnL         .......... .......... ....--.... .......... .........T .....TT... .......... ..........

                     90        100        110        120        130        140        150        160
SA23S        TGGGGAGCTG T-AAGT-AAG CTTTGATCCA GAGATTTCCG AATGGGGAAA CCC-AGCATG AGTTATGTCA TGTTATCGAT
rrnJ§        .......... .-....-... .......... .......... .......... ...-...... .......... ..........
rrnJ-V17     .......... .-....-... .......... .......... .......... ...C...... ...-...... ...-......
rrnE-V12     .......... .-....-... ..-....... .......... ......-... ...-...... .......... ..........
rrnE-V2      .......... .-....-... .......... .......... .......... ...-...... ...-...... ..........
rrnF-V30     .......... .-....-... .......... .......... .......... ...-...... .......... ..........
rrnF-V38     .......... T....T.... .......... .......... ...T...G.. A..C...... ......-... .........-
rrnC         .......... .-....-... .......... .......... .......... ...-...... .......... ..........
rrnA         .......... .-....-... .......... .......... .......... ...-...... .......... ..........
rrnH         .......... .-....-... .......... .......... .......... ...-...... .......... ..........
rrnK         .......... .-....-... .......... .......... .......... ...-...... .......... ..........
rrnL         .......... .-....-... .......... .......... .......... ...-...... ...C...... ..........

                               170        180        190
SA23S                    ATGTG-AAT- ACATAGCATA TCAGAAGGCA CACCCG
rrnJ-4                   .....-...T .......... .......... ......
rrnJ-V4,V17,V32          .....-...- .......... .......... ......
rrnJ-V8                  .....-...- ........G. .......... ......
rrnJ-V43F                .....-...T .......... .......... ......
rrnE-V12                 .....-.-AT .......... .......... ......
rrnE-V2                  .....--.AT .......... .......... ......
rrnF-V30                 .....--.AT .......... .......... ......
rrnF-V38                 -.A..TG.AT .......... .......... ......
V40F                     .....--.AT .......... .......... ......
rrnC                     .....-...- .......... .......... ......
rrnH-v7                  .....--.AT .......... .......... ......
rrnH-V27                 .....-.TAT .......... .......... ......
rrnK-V41                 ..--.TG.AT .......... .......... ......
rrnL-V1                  ..C..TG.AT .......... .......... ......
```

FIGURE 11c

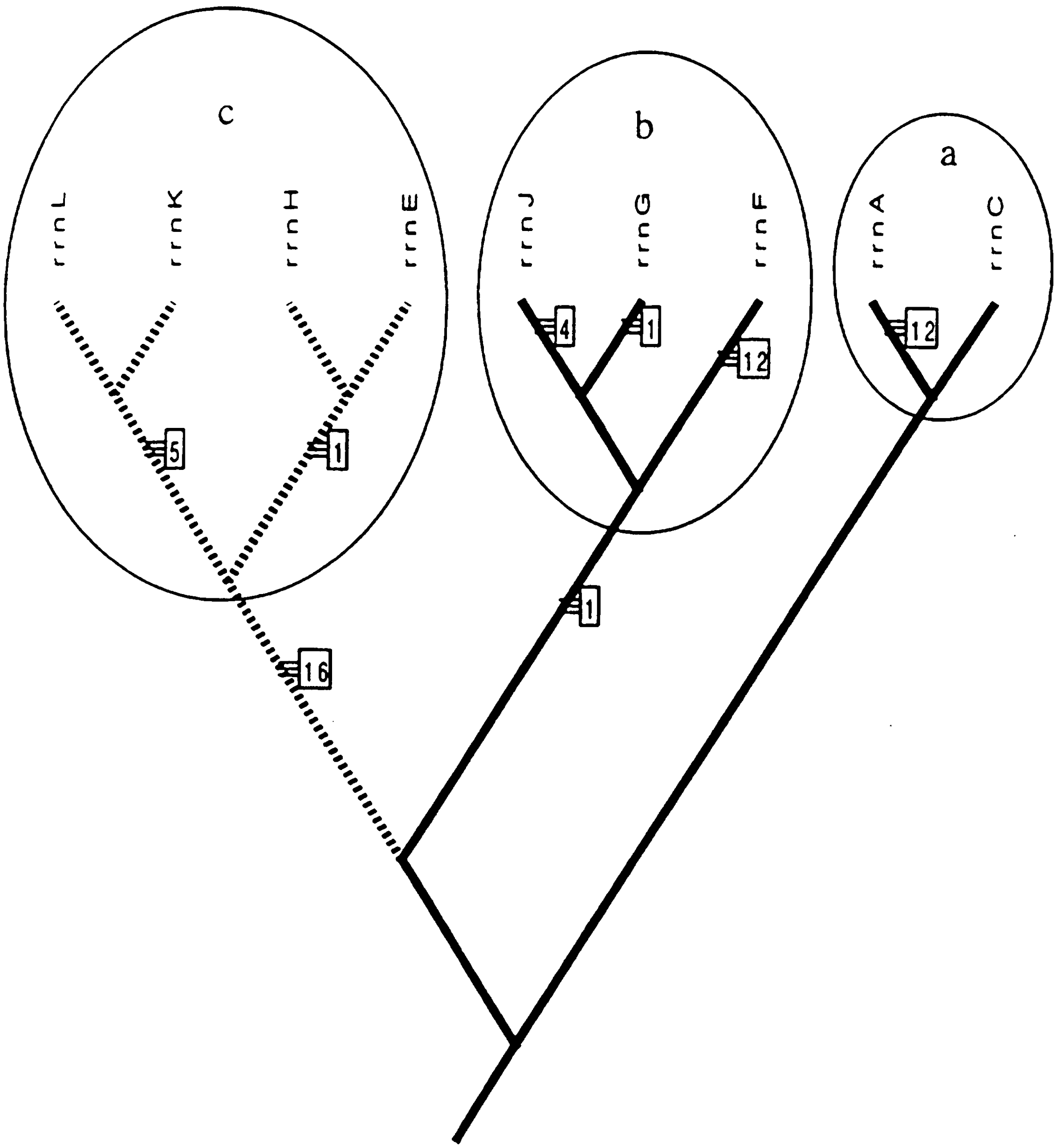
c
b
a
rrnL
rrnK
rrnH
rrnE
rrnJ
rrnG
rrnF
rrnA
rrnC
5
1
4
1
12
12
1
16

FIGURE 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 94/00781

A. CLASSIFICATION OF SUBJECT MATTER	
Int. Cl.[6] C12Q 1/68	
According to International Patent Classification (IPC) or to both national classification and IPC	
B. FIELDS SEARCHED	
Minimum documentation searched (classification system followed by classification symbols) WPAT, Chemical Abstracts. Keywords below.	
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Biotechnology database. Keywords below.	
Electronic data base consulted during the international search (name of data base, and where practicable, search terms used) WPAT keywords: (ribosomal(w)RNA or ribosomal(w)ribonucleic or rRNA or r(w)RNA or r(w)ribonucleic) and (C12Q or G01N or C12N) Chem Abs & BIOT keywords: (ribosomal(w)RNA or ribosomal(w)ribonucleic or rRNA or r(w)RNA or r(w)ribonucleic) and (16s or 23s); (operon# or spacer#) and (ribonucleic or RNA); (ident: or detect: or determ: or assay#) and (pcr or polymerase(w) chain or lcr or ligase(w)chain or amplif: or replicase)	

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X Y	C M Bacot & R H Reeves: "Novel tRNA Gene Organization in the 16s-23s Intergenic Spacer of the Streptococcus pneumoniae rRNA Gene Cluster". Journal of Bacteriology, vol 173, no. 3, pp 4234-4236, July 1991. See entire document.	1-3,14-16 4,8,9
X	T Barry et al: "The 16s/23s Ribosomal Spacer Region as a Target for DNA Probes to Identify Eubacteria". PCR Methods and Applications, vol 1, pp 51-56, (1991).	1-5,10,11, 13-17

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 April 1995 (07.04.95)	13 APRIL 1995 (13.04.95)
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929	Authorized officer RPorter ROBYN PORTER Telephone No. (06) 2832318

C(Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
	See abstract; p 51 column 3 lines 9-26; p 52 column 1 lines 2-4 and 15 - column 2 line 21, column 3 line 7; p 53 column 1 section on "PCR Amplification of the 16s/23s Spacer Sequences"; column 2 lines 8-16 and 45 - page 54 column 1 line 8; p 54 column 1 line 14 - column 3 line 8; p 55 column 1 lines 13-22; p 56 column 1 lines 4-26.	
X	EP, A1, 452 596 (N V INNOGENETICS S A) 23 October 1991 (23.10.91), priority date 18 April 1990 (18.10.90), Int Cl^5 C12Q 1/68, C07H 21/04. See p 2 lines 1-3, p 3 lines 26-44, p 5 lines 39-45 and lines 52 to p 6 line 5, lines 51-53, p 10 lines 41-52, p 11 lines 1-48, p 14 lines 5-8, 13-17, 20-28, p 15 lines 1-13, p 16 lines 5-15, 33-36, p 18 lines 54-p 19 line 9, abstract, claims and examples.	1-5,10, 14-17
X	A K East et al: "Analysis of DNA encoding 23s rRNA and 16s-23s rRNA intergenic spacer regions from <u>Plesiomonas</u> <u>shigelloides</u>". FEMS Microbiology Letters, vol 95, pp 57-62 (1992). See entire document, esp. p 57 summary, p 58 section 3.2.	1,3,5, 15,17
X	T UEMORI et al: "Amplification of the 16s-23s Spacer region in rRNA Operons of Mycoplasmas by the Polymerase Chain Reaction". System. Appl. Microbiol. vol 15, pp 181-186 (1992). See Summary, p 181 column 1 last sentence, column 2 first sentence, p 182, column 1 "3. PCR", "6. Primer preparation", Results, section 1 on "Amplification of sequences", p 185 column 1 lines 6-12, column 2 lines 3-11.	1-5,10, 14-17
X	T Nakagawa et al: "<u>Acholeplasma</u> <u>laidlawii</u> Has tRNA Genes in the 16s-23s Spacer of the rRNA Operon". Journal of Bacteriology, vol 174, no 24, pp 8163-8165 (1992). See entire document.	1,8-10
X	B Sagredo et al: "Organization of the 16s-23s Intergenic Spacer Region of the Two rRNA Operons from <u>Thiobacillus</u> <u>ferrooxidans</u>". Geomicrobiology Journal, vol 10, pp 239-247 (1992). See abstract, p 239 lines 5-15, p 240 lines 1-4, 12-15, p 241 lines 8-12, 15-18, 28-29, p 244 lines 4-8.	1-5,8-11, 14-17
X	R Harasawa et al: "Detection and tentative identifaction of dominant mycoplasma species in cell cultures by restriction analysis of the 16s-23s r RNA intergenic spacer regions". Res. Microbiol. vol 144, pp 489-493 (1993). See p 490-p 491 lines 1-8.	1,2,5,8,10, 11,14,17
<u>X</u> Y	V Gurtler: "Typing of <u>Clostridium</u> <u>difficile</u> strains by PCR-amplification of variable length 16s-23s rDNA spacer regions". Journal of General Microbiology, vol 139, pp 3089-3097 (1993). See entire document.	<u>1-7,10-19</u> 8,9
<u>X</u> Y	A K East & M D Collins: "Molecular Characterization of DNA encoding 23s rRNA and 16s-23s rRNA intergenic spacer regions of <u>Aeromonas</u> <u>hydrophila</u>". FEMS Microbiology Letters, vol 106, pp 129-133 (1993). See abstract, p 129 column 2 lines 12-p 132 column 1 line 3, p 132, column 1 "PCR amplification", column 2 lines 25-28, 36-39 and Figure 2.	<u>1-3,10,14,15</u> 4,8,9,16
X	A Wada et al: "Molecular Cloning and Mapping of 16s-23s rRNA Gene Complexes of <u>Staphylococcus</u> <u>aureus</u>". Journal of Bacteriology, vol 175, no 22, pp 7483-7487 (1993). See p 7483, column 1 line 15-18, 30-38; p 7486, column 2 line 56-61.	1,13,14

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X	L M C Hall: "Are point mutations or DNA rearrangements responsible for the restriction fragment length polymorphisms that are used to type bacteria?" Microbiology, vol 140, pp 197-204 (1994). See p 198, column 1 lines 19-21 and 29-31, p 199, column 1 lines 9-12, p 201, column 2 lines 3-10, p 202, column 1 lines 1-22, column 2 last paragraph, p 203 column 1 last 2 lines - line 1 of column 2.	1-4,8-12, 14-16
X	M F Minnick et al: "Characterization of the 16s-23s rRNA intergenic spacer of Bartonella bacilliformis". Gene, vol 143, pp 149-150 (1994). See entire document especially p 150.	1,2,8-10, 14
X	S Gill et al: "Identification of variability of ribosomal DNA spacer from Pseudomonas soil isolates". Can. J. Microbiol., vol 40, pp 541-547 (1994). See p 542, column 1 last 10 lines - column 2 line 8, p 543, column 1 sections on "Oligodeoxynucleotide primers ..." and "Cloning and DNA sequence ..."; column 2 last 6 lines to p 544 column 1 line 2, column 2 lines 2-24, p 546, column 2 lines 4-19.	1-3,10-15
X	L Dolzani et al: "Typing of Staphylococcus aureus by amplification of the 16s-23s rRNA intergenic spacer sequences". FEMS Microbiology Letters vol 119, pp 167-174 (1994). See abstract, p 168, column 1 lines 28-33, column 2 last 2 lines, p 169, column 1 lines 1-3, column 2 first sentence of results, p 171, column 2 "Discussion" lines 1-9, p 172, column 1 lines 22-28.	1,3-5, 10-13,15-17
X Y	N W Kim & V L Chan: "Isolation and Characterization of the Ribosomal RNA Genes of Campylobacter jejuni". Current Microbiology, vol 19, pp 247-252 (1989). See entire document.	14 1
X Y	G de Lanversin & D T N Pillay: "Primary structure and sequence organization of the 16s-23s spacer in the ribosomal operon of soybean (Glycine max L) chloroplast DNA". Theor. Appl. Genet., vol 76, pp 443-448 (1988). See summary, p 443 column 1 last 3 lines to column 2 line 5, p 445, Table 1, column 1 line 7 - column 2 line 14, p 448, column 1 lines 6-11.	14-16 1-4,8-11
X Y	O Massenet et al: "Sequence organization of the chloroplast ribosomal of Spinacia oleracea including the 3' end of the 16s rRNA and the 5' end of the 23s rRNA". Plant Molecular Biology, vol 10, pp 53-63 (1987). See p 53, sentence spanning columns 1 and 2, p 54 column 2 lines 6-10 of "Results and discussion", p 55, column 1, lines 5-8 and 12-19, Figures 2 and 3.	14-16 1-4,8-11
X Y	M Zacharias & R Wagner: "Functional characterization of a putative internal promoter sequence between the 16s and the 23s RNA genes within the Escherichia coli rrnB operon". Molecular Microbiology, vol 3 number 3, pp 405-410 (1989). See p 405, column 2 lines 29-32 and 35-39, p 408, column 1, "Discussion" lines 4-9 and 14-18.	14-16 1-4
Y	G de Lanversin et al: "Sequence studies on the soybean chloroplast 16s-23s rDNA spacer region". Plant Molecular Biology, vol 10, pp 65-82 (1987). See entire document.	1-4,8-11,14-16
Y	D J Lane: "16s/23s rRNA sequencing". Nucleic Acid Techniques in Bacterial Systematics, pp 115-175 (1991). See entire chapter.	3-5,15-17

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
Y	M J Wolcott: "Advances in Nucleic Acid-Based Detection Methods". Clinical Microbiology Reviews, vol 5 no 4, pp 370-386 (1992). See entire document.	10
Y	E Edmond et al: "A Ribosomal DNA Fragment of Listeria monocytogenes and Its Use as a Genus-Specific Probe in an Aqueous-Phase Hybridization Assay". Applied & Environmental Microbiology, vol 59, no 8, pp 2690-2697 (1993). See entire document.	1-4,14-16
Y	M Y L de Wit & P R Klatser: "Mycobacterium leprae isolates from different sources have identical sequences of the spacer region between the 16s and 23s ribosomal RNA genes". Microbiology, vol 140, pp 1983-1987 (1994). See p 1984, column 1 1st paragraph of "PCR", column 2, 1st paragraph of "SSCP", p 1986, column 1 lines 14-19.	1,10,11

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
EP	452596	AU	77550/91	CA	2080812	EP	525095
		HU	63463	JP	5504889	WO	9116454

END OF ANNEX